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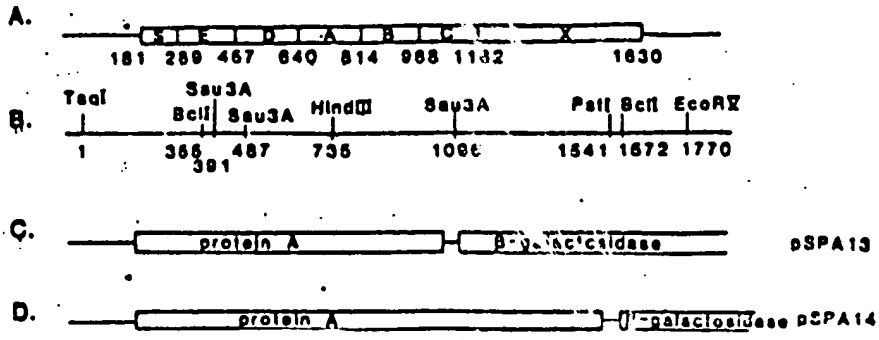

(57) Abstract

A method of producing and selectively isolating a desired protein or polypeptide or derivative thereof by constructing a recombinant vector comprising a DNA sequence coding for said desired protein or polypeptide operatively linked to a DNA sequence coding for protein A or an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, such that said DNA sequences together code for an IgG-binding fusion product between said desired protein or polypeptide and said protein A, active polypeptide fragment thereof or macromolecule; transforming a compatible host with said recombinant vector such that the combined DNA sequences coding for said fusion protein or polypeptide can be expressed by the host, and culturing the transformed host in a suitable growth medium to produce said fusion protein or polypeptide; selectively isolating said fusion protein or polypeptide by adsorption to an IgG-supporting carrier material; and optionally desorbing said fusion protein or polypeptide from said IgG-supporting carrier, said fusion protein or polypeptide coded for by said combined DNA-sequence optionally comprising a unique cleavage site between said protein A part and said desired protein or polypeptide part, said desired protein or polypeptide part then being cleaved off from the rest of the fusion protein or polypeptide either while the latter is adsorbed to the IgG-supporting carrier or after desorption thereof from the carrier. Also a hybrid vector for use herein, a method and an expression vector for its preparation and a host organism transformed by said hybrid vector are disclosed.

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METHOD OF PRODUCING AND SELECTIVELY ISOLATING PROTEINS AND POLYPEPTIDES, RECOMBINANT AND EXPRESSION VECTOR THEREFOR AND FUSION PROTEIN ABLE TO BIND TO THE CONSTANT REGION OF IMMUNOGLOBULINS

The present invention relates to a method of preparing protein and polypeptide products with high purity through recombinant DNA technology, and more particularly to the utilization of such technology to prepare novel gene products comprising desired proteins or polypeptides, which novel gene products are easily refinable, and optionally converting such gene products into the desired proteins or polypeptides. The invention also relates to such novel gene products.

The relatively new recombinant DNA technology or so-called genetic engineering, whereby novel recombinant DNA structures may be constructed from DNA segments derived from different biological sources and introduced into a prokaryotic or eukaryotic host cell to produce the corresponding protein or polypeptide, has made it possible to produce a great number of proteins which can otherwise only be obtained from natural sources and at considerable costs. Well-known examples are insulin and the growth hormone somatostatin. The proteins produced by the host cells are either trapped within the cells or secreted into the surrounding growth medium. In the former case the cells must be ruptured to permit the desired protein to be isolated, whereas in the latter case it can be separated from the growth medium. Even in case of secreted proteins, however, the preparation from which the protein is to be isolated is relatively complex containing a variety of other substances, and despite efficient separation techniques both the purity and yield of the desired protein may be low.

The present invention provides a solution to the above mentioned problem through a method based upon recombinant DNA technology which permits desired proteins and polypeptides to be produced with extreme purity. According to the invention this is achieved by utilizing the unique binding properties of protein A from Staphylococci in combination with gene fusion technology as will be explained below.

Protein A is known as a cell wall component of the bacterium Staphylococcus aureus, hereinafter called S. aureus, and it is characterized by a specific serological reaction with mammal immunoglobulins. In contrast to the normal antigen-antibody reactions, however, protein A binds to the Fc-portion of all subclasses of human immunoglobulins type G, or IgG, except IgG_3, leaving the Fab-portion thereof free for antigen and hapten coupling. This property has given protein A a widespread use in both quantitative and qualitative immunochemical techniques. Covalently bound to a carrier protein A is thus an excellent immunosorbent for the isolation of IgG. The exact structure of protein A may

vary depending on its origin. It has a molecular weight of about 42,000 and a markedly extended shape. The N-terminal part of the molecule comprises four or five highly homologous IgG-binding units, while the C-terminal part lacks Fc-binding ability. As used in the following description and claims, the term "protein A" is, however, not restricted to the above defined staphyloccal protein, but means any macromolecule having analogous immunological and biological activities to the protein A produced by staphylococci, such as the natural strains of S. aureus, including any mutants thereof. Similarly "active fragments of protein A" or "active derivatives of protein A" are meant to comprise any polypeptide fragments or derivatives, respectively, of protein A as well as oligomeric forms of immunoreactive macromolecules or active fragments thereof or other macromolecules that are capable of binding to the constant regions of at least one immunoglobulin.

In our International patent application PCT/SE83/00297 (Swedish patent application No. 8204810-9), the disclosures of which are incorporated herein by reference, the isolation and characterization of the gene coding for staphylococcal protein A as well as the expression thereof in Escherichia coli, hereinafter called E. coli, are described. An E. coli strain transformed with a plasmid containing this staphylococcal protein A gene has been deposited on July 12, 1982 with the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, Federal Republic of Germany, under No. DSM 2434. By using, e.g., this protein A gene containing plasmid, named pSPA1, protein A genetic material for the purposes of the present invention may be obtained.

It is assumed that the specific terms relating to gene technology, which will be used in the following description and claims, are well-known and accepted in the art. Definitions of a selection thereof may, however, be found in, for example, the above mentioned International patent application PCT/SE83/00297.

Gene fusion is a procedure wherein the coding sequence of two or more genes are spliced together to form a combined gene which on expression in a suitable host organism will produce a fusion product wherein the separate proteins or polypeptides coded for by the respective genes are fused together into a single molecule. The gene fusion technique is of growing importance and has so far been used to study various biological problems, such as protein transport mechanisms, plasmid replication and gene expression. Extensive use in this respect has been made of especially the E. coli lac Z gene coding for the enzyme β-galactosidase.

In accordance with the present invention gene fusion is used to combine a first DNA-sequence coding for protein A or an active polypeptide fragment thereof with a second DNA-sequence coding for a desired protein or polypeptide

into a functional gene capable of expressing the fusion product of said desired protein or polypeptide and the protein A component. Due to the IgG-binding ability of the protein A part, the produced protein or polypeptide can easily be isolated with high efficiency by conventional affinity chromatography utilizing immunoglobulin of type IgG immobilized to a suitable carrier. The carrier-bound fusion product may be used as such, e.g. if the desired protein is an enzyme, or it may be released from the carrier, either as a whole including the protein A part, or only the desired protein or polypeptide part thereof through cleavage with a suitable agent as will be further described below.

A basic aspect of the present invention is thus the provision of a recombinant DNA cloning vehicle or vector comprising a DNA sequence coding for a desired protein or polypeptide operatively linked to a DNA sequence coding for protein A or an active polypeptide fragment thereof, such that said DNA sequences together code for an IgG-binding fusion product of said desired protein or polypeptide and said protein A or active polypeptide fragment thereof. In order to be capable of transforming (which is also meant to include the case that the vector is a bacteriophage) a host organism to produce said fusion product, the vector in conventional manner further comprises a replicon and a promoter for the combined fusion product coding DNA sequence. For purposes which will be further elucidated below said combined DNA sequence may comprise a sequence coding for an appropriate cleavage site between the DNA sequences coding for the desired protein and protein A, respectively, such that the protein A part of the fusion molecule may be cleaved off as mentioned above. A more detailed description of the recombinant vector according to this aspect of the invention as well as the construction thereof will be described in more detail further on.

By transforming a compatible host organism with said vector to permit expression of the above combined DNA sequence and culturing the host in a nutrient medium the corresponding IgG-binding fused protein or polypeptide will be produced. Although bacterial hosts, such as strains of, for example, Escherichia, Bacillus and Staphylococcus, are preferred for the purposes of the invention, it is, of course, also within the scope thereof to use other hosts, such as yeasts and other fungi, plant cells in culture, etc. The transformation of the hosts may be effected with well-known methods.

Due to IgG-binding ability of the protein A moiety of the fusion molecule produced by the cultured host-organism the fusion molecule can be very efficiently isolated from the cell culture by means of IgG immobilized to a suitable carrier. If the fusion product is secreted into the surrounding medium the binding to the carrier may be performed directly from the medium. If, on the other hand,

the fusion product remains within the cells the latter have to be ruptured before such immunosorbance can be effected. Rupture of the cell walls may be effected in conventional manner by, e.g., high pressure, ultrasonication, homogenization, shaking with glass-beads etc. In cases where the product is trapped within the periplasmic space between two cell membranes, as in gram-negative bacteria, an osmotic shock procedure may be used to release the product into the suspension medium. Any other treatment of the cultured cells or the growth medium prior to the IgG-aided isolation of the fusion product is, of course, also within the scope of the invention.

In conventional manner the immobilization process may be performed batch-wise with the IgG-coupled carrier slurried in a suitable medium, or on a column of the activated carrier. IgG-coupled carriers for chromatographic use, e.g. IgG-Sepharose® (Pharmacia AB, Sweden) are commercially available and may advantageously be used for the purposes of the invention. However, any conventional carrier material to which IgG can be sufficiently coupled for the present purposes may be used. The methods for coupling or immobilizing IgG to such carrier materials is well-known and need not be described in any detail herein.

Release or desorption of the fused protein or polypeptide which is bound to the IgG-carrier may be effected by conventional methods, such as lowering the pH, e.g. by means of glycine buffer (pH 3.0), treatment with high salt concentrations or chaotrophic ions, or by competitive elution using excess soluble protein A or IgG or fragments thereof to displace the fusion protein or polypeptide from the IgG-carrier adsorbent. The choice of desorption method should, of course, be made with regard to the particular desired protein or polypeptide, such that a desired activity thereof is not lost or considerably reduced thereby. From the resulting eluate the fusion protein or polypeptide may readily be isolated and, if desired, subjected to further purification steps, such as gel filtration, ion exchange etc.

The purified fusion protein or polypeptide obtained may in itself be a valuable product as will be described below, and another aspect of the present invention is therefore the provision of a method of producing a highly purified fused protein or polypeptide product comprising the steps of transforming a compatible host with the above vector, culturing said host in a nutrient medium, isolating said fused protein or polypeptide from said host culture by selective binding thereof to an IgG-supporting carrier, and optionally releasing the fused protein or polypeptide from the carrier, as well as such an isolated fused product obtained thereby.

One valuable use of such a fusion product is when the protein fused to

the protein A part is an enzyme. In such cases the IgG-binding activity of the fusion product is utilized for immobilizing the enzyme to a carrier material having IgG coupled thereto. Such an enzyme system offers several advantages. Since the enzyme is bound to the carrier via the protein A-IgG coupling, all the enzyme molecules will be bound to the carrier in exactly the same way and maximum activity thereof will thus be obtained. When the enzyme activity has decreased to an unacceptably low level, such a system can easily be regenerated by conventionally desorbing the enzyme from the carrier through a pH change, e.g. glycine buffer treatment, and then binding fusion product containing active enzyme thereto. The binding or adsorption of the fused enzyme in question to the IgG-coupled carrier may be effected either directly from the appropriately pre-treated cells or cell medium, or in purified state after adsorption and desorption from another IgG-coupled adsorbent.

Immobilization of enzymes as above may be applied to such enzyme systems which are already used industrially as well as to enzyme systems not yet commercialized, as long as the DNA fragment coding for the selected enzyme is available. As examples of such enzyme systems may be mentioned amino-acid acylase, glucose-isomerase, penicillin-amidase, aspartase, fumarase, β-galactosidase, alkaline phosphatase, etc.

Another case when the IgG-binding ability of the fused protein or polypeptide is desirable is, for example, for providing certain protein A conjugates which may be used in the well-known variant of immuno-chemical analysis named ELISA (enzyme linked immunosorbent assay). Two examples of such conjugates, which are frequently used and also are commercially available, are β-galactosidase and alkaline phosphatase. When these conjugates are prepared in conventional manner, i.e. by chemically binding protein A to the respective enzyme, only part of the two components will be correctly bonded to each other, the resulting conjugate mixture thus containing a relatively high proportion of inactive or poorly active conjugates. In contrast thereto, the corresponding conjugates prepared according to the present invention in the form of a fused gene product will always have the correct coupling relationship between protein A and the enzyme and consequently always maximum and definable activity.

Still another case when the fused protein or polypeptide obtained may be used is when the protein A residue thereof does not inactivate or otherwise prevent the intended use of the desired protein or polypeptide fused to the protein A part. In such a case the fusion product may well be used instead of the respective pure protein or polypeptide, and it will thus not be necessary to cleave off the protein A part therefrom as will be described below as a further aspect of

the invention.

The protein part of the fused protein or polypeptide may under certain conditions be cleaved off, the pure desired protein or polypeptide thereby being obtained. In another aspect the present invention therefore provides a method of producing a desired protein or polypeptide of high purity comprising the steps of transforming a compatible host with the above mentioned vector, culturing said host in a nutrient medium, isolating said fused protein or polypeptide from the cell culture by selective binding to an IgG-supporting carrier, and cleaving off the desired protein or polypeptide from the protein A part of said fused protein or polypeptide, either directly from the carrier bound fusion product or after desorption thereof from the carrier.

A necessary condition to permit such cleavage of the fused protein or polypeptide is, of course, that it contains a unique cleavage site which may be recognized and cleaved by suitable means. Such a cleavage site may be a unique amino-acid sequence recognizable by chemical or enzymatic means and located between the desired protein or polypeptide and protein A sections, respectively, of the fused product to be produced. Such a specific amino acid sequence must not occur within the desired protein or polypeptide and preferably not in the protein A part of the fusion product. Examples of enzymatic agents include proteases, such as collagenase, which in some cases recognizes the amino acid sequence NH_2–Pro–X–Gly–Pro–COOH, wherein X is an arbitrary amino acid residue, e.g. leucine; chymosin (rennin), which cleaves the Met–Phe bond; kallikrein B, which cleaves on the carboxyl side of Arg in X–Phe–Arg–Y; enterokinase, which recognizes the sequence X–$(Asp)_n$–Lys–Y wherein n=2–4, and cleaves it on the carboxyl side of Lys; thrombin which cleaves at specific arginyl bonds. Examples of chemical agents include cyanogen bromide (CNBr), which cleaves after Met; hydroxylamine, which cleaves the Asn–Z bond, wherein Z may be Gly, Leu or Ala; formic acid, which in high concentration (~70%) specifically cleaves Asp–Pro. Thus, if the desired protein or polypeptide does not contain any methionine sequences, which is the case for, e.g., the hormone somatostain, the cleavage site may be a methinonine group which can be selectively cleaved by cyanogen bromide. Often it may be preferred to use chemical cleaving agents because protease recognition sequences may be sterically hindered in the produced fused protein. The techniques for introducing the corresponding DNA sequences coding for such cleavage susceptible peptide units or residues into the DNA sequence coding for the fused protein or polypeptide are well-known per se in the art and need not be discussed in any detail herein. In case a specific cleavage sequence which does not occur in the desired protein, occurs in the protein A molecule,

this amino acid sequence may, without changing the activity of the protein A part, be converted into sequences which are not recognized and cleaved by the particular cleavage means by methods known per se in the art.

As mentioned above the cleavage may be effected either with the fusion product bound to the IgG-coupled carrier or after desorption therefrom. A batch-wise procedure may be carried out as follows. The carrier, e.g. IgG-Sepharose® (Pharmacia AB, Sweden) having the fusion protein or polypeptide bound thereto is washed with a suitable medium and then incubated with the cleaving agent, such as protease or cyanogen bromide. After separation of the carrier material having the protein A residue bound thereto, a solution containing the desired protein or polypeptide and the cleavage agent is obtained, from which the former may be isolated and optionally further purified by conventional techniques such as gel filtration, ion-exchange etc.

In case of the carrier being in column form and the fusion protein or polypeptide comprising a protease recognition site, the cleavage procedure may be performed in the following way. The column of carrier having the fusion protein or polypeptide bound thereto is washed with a suitable medium, and then eluted with an appropriate agent which is gentle to the desired protein or polypeptide to be produced as mentioned hereinbefore. Such an agent may, depending on the particular protein or polypeptide, be a pH-lowering agent, such as, e.g. a glycine buffer, or a protein A solution (competitive elution). The eluate, containing the pure fusion protein or polypeptide together with the cleavage agent, is then passed through a second column comprising the immobilized protease, e.g. collagenase when the cleavage site is a collagenase susceptible sequence. When passing therethrough the fusion protein or polypeptide is cleaved into the desired protein or polypeptide and a protein A residue. The resulting solution is then passed through the same or another IgG-coupled column than used above, whereby the protein A component of the solution is adsorbed and the resulting through-flow is a pure solution of the desired protein or polypeptide. When the desorption agent is a protein A solution, the protein A adsorbed in the last step may be eluted and recycled to cleavage solution, the system then being a regenerative system with respect to protein A.

By means of the above aspect of the present invention a desired protein or polypeptide may readily be obtained with extreme purity and high yields using only a small number of process steps. Such highly purified forms of desired proteins and polypeptides are, for example, excellently suited for the production of anti-bodies through immunisation of an animal, such as rabbit. Another possible application is the combination thereof with the so-called hybridoma technique for the production of monoclonal antibodies.

Desired proteins and polypeptides which can be produced with extreme purity in accordance with the present invention aspect are, for example, enzymes, such as various oxido-reductases, transferases, hydrolases, lyases, isomerases and ligases; hormones, such as parathyroid hormones, growth hormone, gonadotropins (FSH, luteinizing hormone, chorionogonadotropin and glycoproteins), insulin, ACTH, somatostatin, prolactin, placental lactogen, melanocyte stimulating hormone, thyrotropin, parathyroid hormone, calcitonin, enkephalin and angiotensin; and other proteins, such as serum proteins, fibrinogen, fibronectin, prothrombin, thromboplastin, globulin, e.g. gamma-globulins or anti-bodies, heparin, coagulation factors, complement factors, plasma proteins, oxytocin, albumins, actin, myosin, hemoglobin, ferritin, cytochrome, myoglobin, lactoglobulin, histones, avidin, thyroglobulin, interferon, transcortical kinins, etc., and as suggested above peptide antigens for use in making vaccines.

As appears from the above a crucial part of the present invention is the provision of the recombinant DNA structure or vector comprising the combined gene coding for the present fusion protein or polypeptide and capable of transforming a host cell to permit expression thereof and production of the fusion product. The present invention is meant to encompass any such vector irrespective of how it has been obtained using, for example, various restriction enzyme cutting, ligating, transforming and screening techniques well-known in the art as well as any appropriate vector materials and host-organisms. Thus, the DNA sequence coding for the desired protein or polypeptide may be inserted into a suitable vector and the protein A coding DNA sequence inserted subsequently, or vice versa; or the two DNA sequences may be introduced simultaneously into the vector. It is also possible to insert the respective DNA sequences in parts thereof into the vector. Further the two DNA sequences may be arranged with either the protein A coding sequence or the sequence coding for the desired protein or polypeptide at the 5'-end or start of the combined gene. The special techniques for accomplishing such insertions and combinations with maintained correct reading frames, including the provision of suitable restriction sites therefore, are well-known per se in the art.

The source of the DNA sequence coding for protein A or an active polypeptide thereof may be any structure from which the corresponding gene or DNA-segment may be obtained such as a protein A gene containing plasmid. A suitable source is, for example, one of the protein A gene containing plasmids pSPA1, pSPA3 and pSPA5 as constructed and described in our International patent application PCT/SE83/00297 (Swedish patent application 8204310-9). From such a vector the

whole or an appropriate part of the protein A gene may be cut out with suitable restriction enzymes corresponding to suitably located restriction sites in or close to the gene. The extent of the protein A gene to be included in the combined gene coding for the fusion product should be sufficient for imparting IgG-binding activity to the fusion product, which usually means at least a major part of the gene segment coding for the IgG-binding part of the protein A molecule. As will appear from the experimental part below, it may, however, at least in certain cases, be favourable to also include at least part of the gene segment coding for the non-IgG-binding part of the molecule. This part of the molecule will then serve as a spacer between the IgG-active part and the desired protein part of the fusion product.

The source of the DNA sequence coding for the desired protein or polypeptide, which may be of prokaryotic as well as eukaryotic origin, may likewise be any structure from which a corresponding gene or gene segment may be obtained. A suitable source is a plasmid containing such a gene or gene segment. From such a plasmid an appropriate part of the gene in question, i.e. that codes for a sufficient part of the corresponding protein or polypeptide to have the desired activity, may be cut out with suitable restriction enzymes corresponding to suitably located restriction sites in or close to the gene.

The origin of the vector part of the recombinant vector of the invention is preferably a plasmid but it may also be of viral or phage origin. The particular choice of vector depends on the host-organism to be transformed. As mentioned above the latter may be selected from bacteria, fungi, plants and algae. The preferred host is, however, bacteria, and bacteria susceptible to transformation comprise, e.g. members of Enterobacteriaceae, such as strains of E. coli and Salmonella, Bacillaceae, such as Bacillus subtilis, Pneumococcus, Streptococcus, Staphylococcus, Micrococcus and Hemophilus.

When constructing a recombinant DNA vector of the invention it is preferable to first construct an expression or fusion vector comprising a functional DNA sequence coding for protein A or an active polypeptide fragment thereof, and at least one unique restriction site at or near the end of the protein A coding gene. Such a fusion vector may be constructed by providing a suitable vector, e.g. a plasmid vector, containing the whole or a sufficient part of the protein A coding gene as discussed above. A unique restriction site, or preferably a multilinker containing several different restriction sites, is then inserted into the protein A gene after the IgG-binding region but before the stop codon. Such an insertion may, as will appear from the following experimental part, be effected in several steps and plasmids. The resulting fusion vector may then be used for insertion of any DNA sequence coding for a desired protein or

polypeptide. Such a fusion vector is also part of the present invention.

To insert a DNA sequence coding for a desired protein or polypeptide into the fusion vector, it is preferably provided as part of a plasmid. If suitable restriction sites giving complementary ends to those obtained when cutting the fusion vector in one of the unique restriction sites are not present in the gene, such sites may be inserted by conventional methods. They should be inserted as far upstream as possible, i.e. near the 5'-end, in the gene or before the start codon provided that there is no stop codon in-between. By cleaving the fusion vector and the desired gene containing plasmid at the appropriate restriction sites and ligating the mixture, a recombinant vector containing the combined gene may be obtained. Although it may be preferred to cut out the gene coding for the desired protein and insert it into the plasmid, it is also possible to cut the plasmid only at the start of the gene and combine the two plasmids. The above is, however, only a rough example and many variations are possible.

To provide a recombinant vector coding for a fusion protein or polypeptide from which the protein A part may be cleaved off, a synthetic sequence coding for an oligopeptide, which can be recognized by a protease or a peptide cleaving chemical agent, may be inserted between the two fused genes or gene segments. Such insertions may be performed with conventional methods. A proviso is, of course, that the fusion protein or polypeptide, or at least not the desired protein, does not contain other peptide sequences that may be cleaved by the protease or chemical agent.

For the case that a gram-positive bacterium, such as Bacillus subtilis or any staphylococcal species, is to be transformed by the recombinant vector, the control regions of the protein A gene (promoter and ribosome bindning sequence) may advantageously be used. For gram-negative bacteria, such as E. coli, it may be preferable to insert a control region of such origin, e.g. from the E. coli phage lambda.

Apart from the fact that the control regions of the protein A gene function well in gram-positive bacteria, these hosts having a single membrane are favourable from another point of view, viz. that the signal peptide, coded for by the signal sequence of the protein A gene, may serve to secrete the fusion protein or polypeptide into the surrounding medium. In a gram-negative bacterium, such as E. coli, the fusion product will be trapped between the two cell-membranes. Secretion of the product offers great advantages in that the cells need not be ruptured for recovery of the product but can readily be separated and the fusion protein or polypeptide be adsorbed directly from the medium.

As is well-known foreign proteins produced by recombinant DNA

techniques in E. coli may be subjected to proteolytic degradation of the product. Such degradation may be minimized by using a temperature-sensitive repressor which is inactivated at higher temperatures. This permits the gene to be switched off when culturing the bacteria and to be switched on just before the cells are harvested. A DNA sequence coding for such a temperature-sensitive repressor may be introduced into the recombinant vector of the invention by conventional methods.

The invention will now, by way of illustration only, be described in more detail in the following non-limiting examples, reference being made to the accompanying drawings. The disclosures of all the patent and literature references mentioned hereinafter are incorporated by reference herein.

In the drawings:

Fig. 1 is a schematic illustration of a circular restriction map of a plasmid DNA (pSPA1) coding for protein A. The size of the map is given in kilobases starting at the Eco RI restriction site at 12 o'clock, which is a restriction site within the vector pBR322. The positions of the Eco RI, Eco RV, Hind III, Pst I and Bam HI restriction sites are indicated. The junctions between the vector and the inserted DNA are indicated with arrows.

Fig. 2A is a schematic illustration of the protein A coding gene indicating its different regions. Heavy line represents the DNA of the vector pBR322. S is a signal sequence, A–D are IgG-binding regions previously identified, E is a region nearly homologous to A–D, and X is the C-terminal part of protein A which lacks IgG-binding activity.

Fig. 2B is a detailed restriction map of the DNA-sequence corresponding to Fig. 2A, and showing the restriction sites for Taq I, Hind III, Eco RV, Pst I, Bcl I and Sau 3A. The size is given in kilobases starting at the same Eco RI restriction site as indicated in Fig. 1. The junction between the vector pBR322 and the inserted DNA fragment is indicated with an arrow. The restriction sites for Taq I (two) and Sau 3A (one) within the vector sequences have been omitted.

Fig. 3A shows the base sequence around the Sau 3A restriction site at position 1,8 kb in Fig. 2B and Fig. 3B around the Pst restriction site at position 2,1 kb. The amino acid sequence as deduced from the DNA sequence is also shown (the IUPAC amino acid abbreviations are used, J. . Biol. Chem. 24, 527 and 2491 (1966).

Fig. 4 is a schematic illustration of the constructions of plasmids containing the whole or parts of the protein A gene. A few restriction sites are shown. Boxes represent structural genes and the arrows indicate the orientation (from start codon towards stop codon). The replication origin is also indicated by Ori. AMP and TET are the genes coding for ampicillin and tetracycline

resistance, respectively. PROT A is the gene coding for protein A and lac Z' is the gene coding for the N-terminal part of β-galactosidase. (RÜther et al, Nucl. Acids Res. 9, 4087-4098 (1981)).

Fig. 5A and 5B are schematic map illustrations of two fusion vector plasmids. The abbreviations are the same as in Fig. 4. An M13 multilinker has been inserted in the gene coding for protein A at different positions in the two plasmids. The nucleotide sequence and the deduced amino acid sequence of these regions are also shown above the respective plasmid map. Fig. 5A illustrates plasmid pSPA11 and Fig. 5B illustrates plasmid pSPA12.

Fig. 6A and 6B are schematic illustrations of two plasmids containing the genes coding for protein A and β-galactosidase fused together. The abbreviations are the same as in Fig. 4. LAC Z represents the whole gene coding for β-galactosidase except a few nucleotides in the 5'-end thereof. Fig. 6A illustrates plasmid pSPA13 and Fig. 6B illustrates plasmid pSPA14.

Fig. 7 (C and D) is a schematic illustration of the fused protein A and β-galactosidase genes of the plasmids in Fig. 6A and 6B. In the Figure A and B are schematic drawings of the protein A gene corresponding to the restriction map of Fig. 2A and 2B, respectively, and in alignment therewith. The sizes are given in base pairs starting at the Taq I site. (The two Bcl sites at nucleotides 355 and 1572 are also Sau 3A sites).

Fig. 8A is a presentation of the nucleotide sequence around the fusion point of plasmid pSPA13 (Fig. 6A). The restriction sites and the corresponding deduced amino acid sequence are indicated. The origins of the different parts of the sequence are also indicated, and Fig. 8B is a corresponding presentation of the nucleotide sequence around the fusion points of plasmid pSPA14 (Fig. 6B).

Fig. 9 shows the nucleotide sequence of a synthetized oligonucleotide and the corresponding phage mp9/IGF-1 sequence at the point of mutagenesis. "*" indicates a non-complementary base pair. The deduded amino acid sequence after mutagenesis is also shown.

Fig. 10 is a schematic illustration of the construction of shuttle vectors containing the IFG-1 and the protein A genes. A few restriction sites are shown. Boxes represent structural genes and the arrows indicate the orientation (from start codon towards stop codon). The replication origins are also indicated by ORI-E (E. coli) and ORI-S (S. aureus). AMP, TET and CML are the genes for ampicillin, tetracycline and chloramphenicol resistance, respectively. PROT A is the gene coding for protein A and IGF-1 is the gene coding for the modified

human insulin-like growth factor, type 1.

Fig. 11 is a presentation of the nucleotide and the deduced amino acid sequence of the gene fusion between protein A and IGF-1 in plasmid pUN201. Only the DNA-sequence coding for the mature protein A (lacking the region coding for the signal peptide) is shown. Possible amino acid cleavage points with formic acid treatment (Asp-Pro) are underlined. Cleavage sites for Eco RI and Hind III are shown, said cleavage sites representing the end points of a synthetic modified IFG-1 gene.

Fig. 12 is a presentation of the nucleotide sequence, and the corresponding deduced amino acid sequence, around the 3'-end of the protein A gene in plasmid pUN202.

Fig. 13A is a presentation of two DNA-strands representing the IGF-1 gene and flanking sequences, divided into oligomers. The sequence has been provided with a start codon (ATG in block A2), a stop codon (TAG in block A17) and recognition sequences for Eco RI (block A1) and Hind III (block A17).

Fig 13B is a ligation pattern for the IGF-1 gene.

In the following Examples the starting materials, buffers, cell media and routine method steps were as follows.

STARTING MATERIALS

Bacterial hosts. Four strains of E. coli were used in the Examples: HB101, described by Boyer et al, J. Mol. Biol. 41, 459-472 (1969); XAC lac, (Miller et al, J. Mol. Biol., 109, 275-301 (1977); RRI del M15 (Langey et al, Proc. Natl. Acad. Sci., USA, 72, 1254-1257 (1975); JM 83 (Viera and Messig, Gene 19, 259-268 (1982)). Also S. aureus SA113, described by Iordanescu et al, J. Gen. Mic obiol. 96: 277-281 (1976), was used. (The strains are available at the Department of Microbiology (N), Biomedical Centre, Uppsala, Sweden).

Cloning vehicles. The cloning vehicles used in the Examples were

pBR322 as constructed and described by Bolivar et al, Gene 2, 95-113 (1977);

pUR 222 as constructed and described by Rüther et al, Nucl. Acids Res., 9, 4087-4098 (1981);

PTR262 as constructed and described by Roberts, T.M. et al, Gene 12, 123-127 (1980);

pUC8 as constructed and described by Viera and Messig, Gene 19, 259-268 (1982);

pHV14 as constructed and described by Ehrlich, S.D., Proc. Natl. Acad. Sci. USA 70, 3240-3244 (1978);

pSKS104 and pSKS106 as constructed and described by Casadaban, M.J., Martinec-Arias, A., Shapiro, F., and Chou, J., Methods in Enzymology, 100, p. 293-308 (1983);

plasmids pSPA1, pSPA3, pSPA5 and pSPA16 containing the gene coding for

staphylococcal protein A as constructed and described in the International patent application PCT/SE83/00297 (Swedish patent application 8204810-9). Cultures of an E. coli 259 strain containing plasmid pSPA1 and of an S. xylosus KL117 strain containing plasmid pSPA16 have been deposited with the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, Federal Republic of Germany, under No. DSM 2434 on July 12, 1982 and No. DSM 2706 on August 15, 1983, respectively; Phage vectors M13 mp8 and mp9 RFI DNA, supplied by New England Biolabs, Beverly, MA, USA (catalogue No. 408 and 409).

BUFFERS AND MEDIA

Tris-EDTA buffer ("TE"):	0.001 M EDTA and 0.01 M Tris (pH 7.8)
Coating buffer (carbonate-bicarbonate - pH 9.6)	1.59 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3, made up to 1 liter with distilled H_2O
PBS TWEEN: (Phosphate buffered saline plus 0.05% TWEEN®)	8.0 g NaCl, 0.2 g KH_2PO_4, 2.9 g $Na_2HPO_4 \times 12\ H_2O$, 0.2 g KCl, 0.2 ml TWEEN® 20 and 0.2 g NaN_3, made up to 1 liter with distilled H_2O; pH 7.4
Diethanolamine buffer 10%	97 ml diethanolamine, 800 ml distilled H_2O, 0.2 g NaN_3, and 100 mg $MgCl_2 \times 6H_2O$; pH adjusted to 9.8 with 1 M HCl; made up to 1 liter with distilled H_2O
Luria-broth ("LB"):	10 g Difco tryptone, 5 g Difco yeast extract, 0.5 g NaCl, 2 ml 1M NaOH; adjusted to pH 7.0 with 1 M NaOH; 10 ml 20% glucose added after autoclaving.
LA-medium:	Luria broth supplemented with 1% Difco agar
TEB buffer	0,09 M TRIS-borate, 0,09 M boric acid and 0,002 M EDTA.
ONPG buffer	2 mM o-nitrophenyl-β-D-galactoside (ONPG, Sigma product No. N-1127) in 0.1 M potassium phospate buffer, pH 7.3, containing 0.1 M 2-mercaptoethanol and 1 mM $MgCl_2$.
Xgal-medium	LA-medium supplemented with 40 mg/l of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal).
AXI	LA-medium supplemented with 50 mg/l of ampicillin, 40 mg/l of 5-bromo-4-chloro-3-indoyl-β-D-galactoside (Xgal) and 0.1 mM isopropyl-β-D-thiogalactoside (IPTG).

ROUTINE METHODS

Certain procedures were carried out repeatedly in the Examples. Unless otherwise specified, they were done exactly as follows each time that they were carried out.

Transformations. Transformation of E. coli K 12 with plasmid DNA, was performed exactly as described by Morrison, D.A., Methods in Enzymology, Academic Press 68, 326-331 (1979). The transformed cells were selected in a conventional manner on plates by plating for single colonies on LA plates containing suitable antibiotics, i.e. 35 µg/ml of ampicillin or 25 µg/ml of chloroamphenicol.

Isolation of plasmids. Large scale plasmid preparation was performed exactly as described by Tanaka, T. and Weisburn, B., J. Bacteriol. 121, 354-362 (1975). For scoring a large number of clones for plasmids the "mini alkali method" was used exactly as described by Birnboim, H.C. and Doly, J., Nucl. Acids Res. 7, 1513-1523 (1979).

Gel elution. DNA fragments were eluted from either polyacrylamide or agarose gel pieces exactly as described by Maxam et al, P.N.A.S. 74, 560-564 (1977).

DNA sequencing. DNA fragments were 5'-end labelled, and their DNA sequences were determined exactly as described by Maxam et al, supra. The 5'-end of endonuclease generated DNA fragments was labelled with (γ-^{32}P) ATP (New England Nuclear, USA; 2700 Ci/mmol) using T4 polynucleotide kinase (Boehringer, Mannheim, West Germany).

Restriction enzyme digestion of DNA. DNA was cleaved with conventional restriction enzymes purchased from New England Biolabs, Beverly MA, USA. The restriction enzymes were added to DNA at conventional concentrations and temperatures and with buffers as recommended by New England Biolabs.

Ligating DNA fragments. All DNA fragments were ligated at 14°C over-night with T4 DNA ligase purchased from New England Biolabs, Beverly MA, USA, in a buffer recommended by the supplier.

Agarose gel electrophoresis . 0.7% agarose gel electrophoresis for separating cut plasmid fragments, supercoiled plasmids, and DNA fragments 1000 to 10,000 nucleotides in length was performed exactly as described by Helling et al, J. Vir. 14, 1235-1244 (1974).

Polyacrylamide gel electrophoresis. 8% polyacrylamide gel electrophoresis for the separation of DNA fragments 100 to 4000 nucleotides in length was performed exactly as described by Maxam et al, P.N.A.S. 74, 560-564 (1977).

Preparation of cell lysate for detection of protein A. E. coli clones were grown overnight at 37°C in 50 ml Luria-broth (LB) with ampicillin added at 35 µg/ml. After centrifugation the cells were resuspended in 5 ml Tris-EDTA (0.05 M, pH

8.5, 0.05 M) and centrifuged. The cells were resuspended in 5 ml of the same buffer and lysozyme was added to a final concentration of 2 mg/ml. After 1 hour at 37°C the lysate was centrifuged in a Sorvall SS-34 rotor at 15.000 rpm for 15 minutes. The supernatant was collected and assayed for protein A.

Detection and quantification of protein A from E. coli clones. An ELISA-test (enzyme linked immunosorbent assay) was used for detection and quantification of produced protein A. The test makes use of a special microtiter plate (Titertek, Amstelstad, the Netherlands) having no net charge (neutral), the wells of which are coated with human IgG (Kabi, Sweden). Test samples are then added to allow protein A to bind to the Fc-part of the IgG-molecules. The amount of remaining free Fc-sites is then titrated by adding alkaline phosphatase linked to protein A. After washing of the wells, p-nitrophenyl-phosphate is added as a substrate for alkaline phosphatase.

Assay: The wells of a microtiter plate were filled with 50 µl of a solution of human IgG (Kabi, Sweden) at 500 µg/ml in a coating buffer and the plate was incubated at room temperature for 1 hour. The wells were then washed three times with PBS +0.05% Tween® 20, which was used in all washes in the assay, and 50 µl of the lysate to be tested was added. For quantitative determinations twofold serial dilutions of the lysates in PBS +0.05% Tween® 20 were made. 10 µl of PBS + 0.1% Tween® 20 was then added and incubation was allowed for 1 hour at room temperature. The wells were again washed three times, and 50 µl of protein A-alkaline phosphatase conjugate (prepared exactly as described in Immunochemistry, Pergamon Press 1969, Vol. 6 pp. 43–52) was added. After 1 hour of incubation at room temperature the wells were again washed three times and 100 µl of alkaline phosphatase substrate (Sigma 104 = p-nitrophenyl-phosphate at 1 mg/ml) was added. The enzyme reaction was interrupted after 30 minutes by the addition of 10 µl of 3 M NaOH. The result was determined visually. A positive result, i.e. presence of protein A, is a colour-less reaction mixture, since no free Fc-sites of IgG are available to bind the conjugate. A negative result, i.e. no protein A, is observed as a yellow colour due to the activity of the alkaline phosphatase of the bound conjugate. Quantitative determinations of protein A were made by running serial twofold dilutions of a protein A standard solution of known concentration in parallell with the test samples.

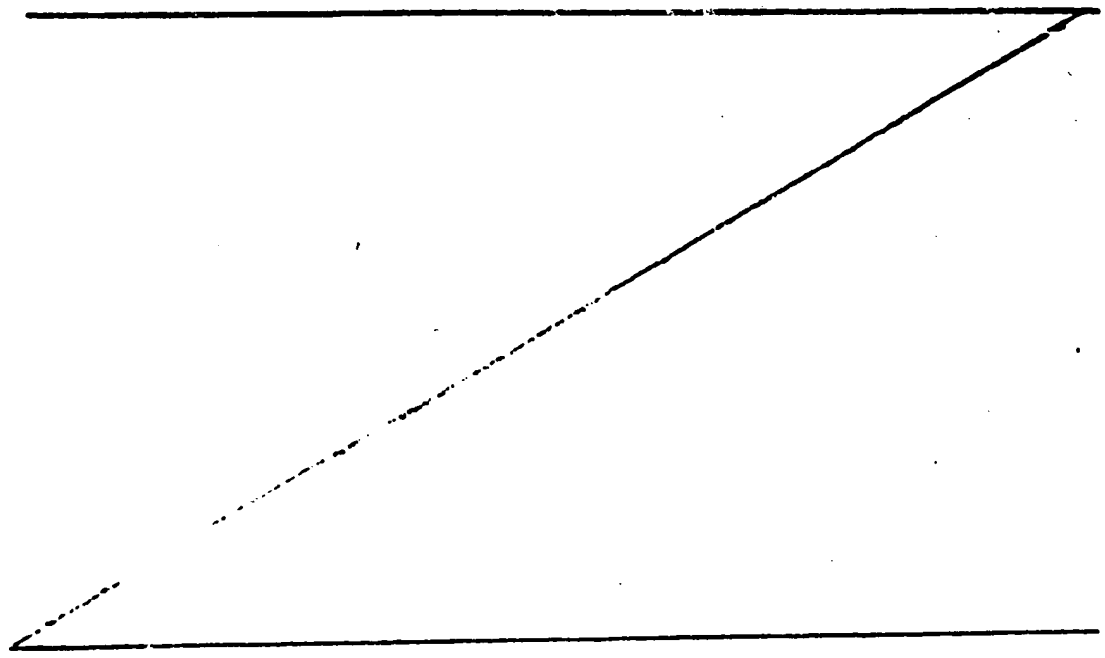
β-galactosidase assay

Recombinants containing a functional lac Z gene were scored by plating on Xgal medium. Cell free β-galactosidase activity was assayed by a colorimetric procedure using o-nitrophenyl-β-D-galactoside (ONPG, Sigma product No. N-1127) as substrate as described by Miller, J. H., Experiments in Molecular

Genetics, Cold Spring Harbor, New York; Cold Spring Harbor Laboratory, 1972, with the following modifications. The assay was performed at +8°C and the activity was measured at 405 nm. One unit of activity represents the change in absorbance at 405 nm per minute. β-galactosidase activities of the fused proteins coupled to IgG-Sepharose® were determined at 3°C, inverting the tube in order to prevent sedimentations.

Phage M13 cloning and sequencing.

All M13 cloning, purifications and sequencing was performed as described in the Instructions/Protocol obtained by the supplier (New England Biolabs, Beverly, MA., USA, catalogue No. 408 and 409).



EXAMPLE I

I. Analysis of the DNA sequence of the protein A gene

In order to make fusions between genes or gene parts it is desirable to know the DNA sequence and its deduced amino acid sequence around the fusion point of the two genes or gene parts to be fused. With the knowledge thereof it will be possible to predict how the linkage should be designed in order to give the correct reading frame in both genes or gene parts and thus possibly the expression of a functional hybrid protein.

In our Swedish patent application No. 8204810-9 (the disclosure of which is incorporated herein by reference) the construction of three plasmids containing the whole structural gene coding for staphylococcal

protein A is described, viz. plasmids pSPA1, pSPA3 and pSPA5. However, only the DNA sequence of the 5'-end of the protein A gene (regions S, E, D and part of A in present Fig. 2A) is disclosed. A preliminary sequence of the whole protein A gene was therefore determined in order to obtain more detailed information of the DNA sequence in the 3'-end of the gene. This sequencing operation was effected as described above under Routine Methods, the DNA source being purified plasmid pSPA3, which is the smallest of the three protein A gene containing plasmids and therefore the least difficult one to sequence. In Fig. 3 the obtained DNA sequences around the Sau 3A restriction site at position 1,8 kb and around the Pst I restriction site at position 2,1 kb in the protein A gene restriction map of Fig. 2B are shown together with the corresponding deduced amino acid sequence . The particular interest in the above two restriction sites for the present purposes will be explained below.

Based upon the DNA sequence obtained it was decided to construct two different gene fusion vectors by inserting an M 13 multilinker (an oligonucleotide containing restriction sites for several restriction enzymes) into the above mentioned Sau 3A site at nucleotide 1096 of Fig. 3A and the Pst I site at nucleotide 1541 of Fig. 3B. These sites are located before and after the repetetive part of region X of the protein A gene (Fig. 2A) which is thought to be involved in the binding of protein A to the peptidoglycan of the cell wall in S. aureus. The possible influence thereof on the fused proteins to be produced on expression of genes fused by means of such fusion vectors could then be determined. The construction of the two gene fusion vectors is described hereinafter.

II. Construction of fusion vector plasmid pSPA11 (Fig. 5A)

In the following steps A-E the construction of a plasmid containing the protein A gene without region X and having a unique Eco RI site inserted at the Sau 3A site at position 1098 (Fig. 7) is described.

A. Subcloning of the 5'-end of the protein A gene from pSPA1 into plasmid pTR262 to obtain plasmid pSPA2 (Fig. 4)

1 µg of plasmid pSPA1 (see Fig. 1) and 1 µg of plasmid pTR262 were cut with restriction enzymes Hind III and Pst I, mixed, treated with T4-ligase and used to transform E. coli HB101. Cleavage, ligation and transformation were effected as described under Routine Methods.

Plasmid pTR262 contains a lambda repressor gene which on expression inactivates the gene for tetracycline resistance. The lambda repressor gene has a Hind III site and insertion of a DNA sequence into the latter therefore inactivates the lambda repressor gene and activates the tetracycline resistance gene. Plasmid pTR262 thus permits positive selection for tetracycline resistant

recombinants.

Colonies containing recombinants were thus selected as being tetracycline resistant. 1 colony out of 20 of these recombinants was discovered to be protein A positive using the ELISA method described under Routine Methods. Restriction analysis indicated that it contained vector plasmid pTR262 having a 2.1 kb protein A gene insert derived from the fragment corresponding to 0.0 to 2.1 kb of the pSPA1 restriction map of Fig. 1 and 2B. This plasmid was designated pSPA2 and is shown schematically in Fig. 1. It has a unique Pst I restriction site at the 3'-end of the protein A gene fragment which will be utilized in the following step E.

B. Preparation of a DNA fragment containing the protein A gene

100 µg of plasmid pSPA5(plasmid vector pHV14 having a protein A gene insert; see Starting Materials above) were cut with restriction enzyme Eco RV for 1 hr at 37°C. This produced two DNA fragments, viz. the inserted DNA fragment containing the protein A gene (2.1 kb) between positions 0.2 kb and 2.3 kb in Fig. 2B and the vector pHV14 (7.2 kb). This digest was heat inactivated, precipitated with ethanol, dissolved in 100 µl of TE and sedimented through a 10-30% sucrose gradient in TE buffer. A Beckman SW40 rotor was used at 5°C, 35,000 rpm, for 20 hrs. The gradient was fractionated into 0.5 ml fractions, each of which was analyzed by agarose gel electrophoresis. The fractions containing the 2.1 kb fragment were pooled, precipitated with 2 volumes of ethanol and dissolved in TE buffer. As appears from Figs. 2A and B the fragment contains, in addition to the whole protein A gene, an E. coli sequence derived from plasmid pBR322 and a staphylococcal gene residue.

C. Preparation of a DNA fragment containing a part of the protein A gene

5 µg of the purified 2.1 kb fragment from step B were cut with restriction enzyme Sau 3A for 1 hr at 37°C. The digest was run on a preparative 8% polyacrylamide gel electrophoresis in TEB buffer. The gel was stained with ethidium bromide (1 µg/ml) and a DNA fragment of approximately 600 base pairs was cut out. This fragment corresponds to the part of the gene between positions 1.15 and 1.8 kb in Fig. 2B. The DNA was eluted overnight at 37°C in 5 ml of TE + 0.3 M NaCl. The eluate was passed over a column containing approximately 300 µl of sedimented DE-52 (Whatman, England) equilibrated with 5 ml of TE. After a 2 ml wash with TE + 0.3 M NaCl the DNA was eluted with two volumes of each 0.5 ml of TE + 0.6 M NaCl. The eluate containing the DNA fragment was diluted with one volume of TE, precipitated with ethanol and dissolved in TE buffer. The resulting purified protein A gene fragment has cohesive ends corresponding to a Sau 3A restriction site and an intermediate Hind III site.

D. Preparation of vector plasmid pUR222

Plasmid pUR222 is a commercially avilable vector containing the gene coding for the enzyme β-galactosidase (lac Z). The gene comprises a multilinker having several restriction sites, such as Pst I, Bam H1 and Eco RI. Since β-galactosidase is easily detectable by enzymatic assays, recombinants having a DNA fragment inserted in one of the restriction sites can easily be scored with the use of appropriate host strains. Often Xgal plates are used (Xgal is a chromogenic substrate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside, which releases a blue indolyl derivative when cleaved by β-galactosidase) upon which β-galactosidase negative recombinants appear as white colonies in contrast to the blue-green colour of colonies containing plasmids without an insert.

To cleave plasmid pUR222 in the β-galactosidase coding gene to provide cohesive ends complementary to the cohesive ends of the protein A fragment of step C for insertion thereof into the plasmid, the Bam H1 restriction site was used. 1 µg of pUR222, supplied by Boehringer-Mannheim, Germany, was digested with the restriction enzyme Bam H1 for 1 hr at 37°C, whereupon the enzyme was inactivated at 65°C for 10 minutes. This cleavage preparation was used in the following step E for ligation with the protein A fragment.

E. Construction of a hybrid plasmid pSPA10 containing pSPA2 and pTR262 (Fig. 4)

200 ng of pUR222 digested with Bam H1, as described in step D, and 200 µg of eluted protein A fragment, as described in step B, were mixed and ligated in a total volume of 20 µl overnight at +14°C. The enzyme was inactivated at 65°C for 10 minutes, precipitated with ethanol and dissolved in TE buffer. The whole DNA-mixture containing i.a. recombinant plasmids having the protein A insert in the β-galactosidase gene was cut with restriction enzymes Hind III and Pst I for 1 hr at 37°C in the recommended buffer for Hind III. This cleaves the recombinant plasmid in the β-galactosidase gene (Pst I) and in the protein A gene (Hind III) producing two fragments, viz. a small fragment consisting of a minor β-galactosidase DNA sequence linked to the part of the protein A gene fragment from the Sau 3A site at position 1.15 kb to the Hind III site in Fig. 2B, and a large fragment consisting of the rest of the recombinant plasmid, which comprises the major part of the β-galactosidase gene linked to the protein A gene fragment from the Hind III site to the Sau 3A site at position 1.8 kb in Fig. 2B. As appears from Fig. 4 the β-galactosidase fragment has an Eco RI restriction site close to the point of fusion with the protein A fragment (the Bam H1 site).

200 ng of plasmid pSPA2 from step A were cut with the restriction enzymes Hind III and Pst I in the same way as above to cleave the plasmid

into (see Fig. 4) three fragments, viz. one fragment extending from the Hind III site located between the Tet-gene and the 5'-end of the protein A gene to the Hind III site within the protein A gene, a protein A gene fragment extending from the latter Hind III site to the Pst I site at the 3'-end of the protein A gene, and a larger fragment of pTR262 origin comprising the rest of the plasmid.

The two digests prepared above were inactivated at 65°C for 10 minutes, mixed and precipitated with ethanol. The DNA was dissolved in ligation buffer and treated with T4-ligase. The desired recombinant plasmid comprises the above mentioned large fragment, obtained on cleavage of the pUR222 recombinant, inserted in pSPA2 between the Hind III site within the protein A gene and the Pst I site and comprising the 5'-end of the protein A gene, one part thereof thus being derived from pSPA2 and the other originating from the pUR222 recombinant. Further, the plasmid is ampicillin and tetracycline resistant and should give blue colour on Xgal plates as will be explained below.

The ligated DNA-mixture was therefore used to transform E. coli RRI del M15. Cleavage, ligation and transformation were effected as described above. Recombinants were plated out on Xgal plates containing ampicillin and tetracycline. One of the clones appeared as light blue, and restriction analysis was performed on its plasmid. This revealed a plasmid, designated pSPA10 (Fig. 4), which consists of parts of plasmid pUR222, plasmid pTR262 and the protein A gene originating from plasmid pSPA1. In plasmid pSPA10 the protein A gene fragment is fused to the lac Z'gene through its Sau 3A site at position 1096 as appears from Fig. 7.

Although plasmid pSPA10 does not contain the whole lac Z gene coding for β-galactosidase but only the gene coding for the α-fragment thereof (lac Z'), it is active in cleaving the Xgal substrate thereby producing blue colour under the above used conditions. This is due to a complementation between the α-fragment coded by the plasmid and a chromosomal gene product containing the carboxy terminal fragment of β-galactosidase resulting in an active enzyme. The E. coli RRI del M15 host strain used above has such chromosomal gene material and therefore complements the α-fragment produced by the pSPA10 plasmid to an active β-galactosidase molecule.

The above described steps A-E thus produced a plasmid vector pSPA10 containing a desired protein A fragment which has a unique Eco RI site adjacent to its downstream end. In order to construct a convenient fusion vector a DNA-linker containing multiple restriction sites was introduced at that site simultaneously with removal of the non-desired fragment (containing the lac Z'gene and the gene for ampicillin resistance) between the Eco RI and Pst I sites, as will be described in the following section III.

F. Construction of fusion vector plasmid pSPA11 containing a multi-linker from phage M 13 mp 8 (Fig. 5A)

1 µg of plasmid pSPA10 from step E and 2 µg of phage vector M13 mp 8 (supplied by New England Biolabs, Beverly, MA, USA) were cut separately with the restriction enzyme Eco RI, precipitated and dissolved in an appropriate buffer for the restriction enzyme Pst I. Digestion with the Pst I enzyme was performed and the two DNA digests were mixed, ligated and used to transform E. coli HB101. Cleavage, ligation and transformation were effected as described above under Routine Methods. The desired recombinants were selected as being tetracycline resistant and ampicillin sensitive. 52 tetracycline resistant clones were picked onto plates containing ampicillin. 3 of these clones were found to be ampicillin sensitive and restriction analysis on one of them revealed the plasmid schematically shown in Fig. 5A. This plasmid, which contains an inserted M13 multi-linker at the end of region C of the protein A gene (position 1.8 kb, Fig. 2B), was designated pSPA11. The deduced amino acid sequence is also indicated providing a guide for obtaining the correct reading frame after gene fusions. Plasmid pSPA11 is a vector well suited for gene fusions with the protein A fragment as will be demonstrated in the following step III.

III. Fusion of plasmid pSPA11 to the E. coli lac Z gene

1 µg of plasmid pSKS104 (containing a unique E. coli RI site) was digested with the restriction enzyme Eco RI. This plasmid, together with the plasmid pSKS106 used below, are examples of a set of plasmids constructed to aid gene fusions between the E. coli lac Z gene and other genes. The hybrid protein produced by such fusions contains, at its carboxyterminus, enzymatically active β-galactosidase (minus a few amino acids at the N-terminus) and can be assayed by the enzymatic activity thereof. 1 µg of plasmid pSPA11 from step 2F (also having a unique Eco RI site) was digested separately with restriction enzyme Eco RI. Both DNA digests were heat inactivated, mixed, ligated and used to transform E. coli XAC lac (which strain lacks the β-galactosidase gene) as described above. Recombinants were scored on Xgal plates containing both tetracycline and ampicillin. Approximately half of these clones were light blue (the cleaved pSKS104 can be inserted in correct or wrong direction with equal probability) and restriction analysis on one of these revealed the plasmid schematically represented in Fig. 6A. This plasmid, designated pSPA13, contains the lac Z gene fused to the protein A gene at the nucleotide of position 1.8 kb in Fig. 2B. This is schematically shown in Fig. 7 and the deduced amino acid sequence over the fusion point is shown in Fig. 8A. Cultures of this clone have been deposited on February 4, 1983 with the collection of the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, Federal Republic of Germany, where it was assigned

No. DSM 2591.

IV Fusion of plasmid pSPA2 to the E. coli lac Z gene

Plasmid pSPA2, as constructed in step IIA above and shown in Fig. 4, contains a unique Pst I site at position 1541 of the protein A gene (see Fig. 7). This plasmid was therefore used for gene fusion of the corresponding protein A gene fragment to the lac Z gene of plasmid pSKS106.

1 µg of plasmid pSPA9 from step 2C and 1 µg of plasmid pSKS106 were cut separately with restriction enzyme Pst I. The resulting DNA-fragments were mixed, ligated and used to transform E. coli XAC lac as described under Routine Methods. Recombinants were scored on Xgal plates containing tetracycline and ampicillin. As in section III above approximately half of these clones were light blue and restriction analysis on one of these revealed a plasmid schematically represented in Fig. 6B. This plasmid, designated pSPA14, contains the lac Z gene fused to the protein A gene at position 2.1 kb in Fig. 2B. This is schematically shown in Fig. 7, and the deduced amino acid sequence over the fusion point is shown in Fig. 8B. Cultures of this clone have been deposited on February 4, 1983 with the collection of the Deutsche Sammlung von Mikroorganismen (DSM), Göttingen, Federal Republic of Germany, where it was assigned No. DSM 2592.

V. Construction of fusion vector plasmid pSPA12 (Fig. 5B)

In order to make fusions of the protein A gene fragment of plasmid pSPA9 more generally possible a corresponding fusion vector as pSPA11 above was constructed by cutting out the lac Z gene from plasmid pSPA14 and retaining the multi-linker sequence preceding the 5'-end of the gene.

This was achieved by cutting 1 µg of plasmid pSPA14 from section IV with restriction enzyme Eco RI, ligating and transforming E. coli XAC lac as described above under Routine Methods and scoring for tetracycline resistance and lack of β-galactosidase activity. Clones were plated on Xgal plates containing tetracycline. Approximately 80% of these colonies were white and restriction analysis on one of these revealed a plasmid schematically shown in Fig. 5B. This plasmid, designated pSPA12, contains the M13 multi-linker at position 2.1 kb in Fig. 2B. The reading frame at the fusion point is shown in Fig. 5B.

VI. Subcloning of the whole protein A coding gene into plasmid pBR322 for construction of plasmid pSPA8 (Fig. 4)

The above constructed fusion vectors pSPA11 and pSPA12 and the corresponding fused genes containing plasmids pSPA13 and pSPA14 all lack any E. coli residues upstream of the protein A gene originating from starting plasmid pSPA1, including the E. coli promoter. In order to construct, for comparative purposes, a plasmid which contains the whole structural gene of protein A,

including the protein A promoter sequence, but which lacks any E. coli promoter upstream thereof, the 2.1 kb protein A fragment from step IIB above was cloned into the plasmid vector pBR322 as follows below.

1 µg of the purified 2.1 kb protein A fragment from step IIB was cut with restriction enzyme Taq I for 1 hr at 60°C to cleave it within the DNA of staphylococcal origin. The enzyme was inactivated by extraction with an equal volume of phenol, followed by repeated ether extraction, and finally the DNA was precipitated with ethanol and dissolved in TE buffer. 1 µg of plasmid pBR322 was cut with restriction enzymes Cla I and Eco RV (which cleave in the same way and thus provide complementary cohesive ends) for 1 hr at 37°C in Bam H1 buffer and then heat inactivated for 10 minutes at 65°C. The DNA samples were mixed, ligated and used to transform E. coli HB101 as described above under Routine Methods. Transformants were streaked out on ampicillin (35 µg/ml). Colonies were picked on plates containing 10 µg/ml of tetracycline and 35 µg/ml of ampicillin, respectively. Transformants that grew on ampicillin but not on tetracycline were considered as recombinants. 4 colonies out of 12 of these recombinants were discovered to be protein A positive using the ELISA method described under Routine Methods. Restriction analysis in which purified plasmid was cut with one, two or three restriction enzymes were performed on one of these clones. The resulting restriction map of this plasmid, designated pSPA8, is shown in Fig. 4. The thus constructed plasmid lacks any E. coli promoter upstream of the protein A gene. The protein A gene fragment is preceded by its own staphylococcal promoter only.

VII. Detection and quantitation of protein A from E. coli clones

To evaluate the protein A activity of the two plasmids pSPA13 and pSPA14 constructed above, comparisons were made with plasmid pSPA8 from section VI above, containing the whole structural protein A gene, and plasmid pSKS106, containing the β-galactosidase gene, with regard to total content of protein A and ability to bind to IgG-Sepharose® columns as follows.

Cell suspensions of 300 ml carrying plasmids pSKS105, pSPA8, pSPA13 and pSPA14, respectively, were separately grown to OD_{550} = 1.0 in LB medium containing 35 µg/ml of ampicillin without added glucose. Each cell culture was then centrifuged at 6000 rpm with a Sorvall GSA-rotor for 10 min. and the cell pellets were washed in 20 ml of TE (0.05 M tris, pH 8.5, 0.05 M EDTA) and again centrifuged as above. Finally the cell pellets were resuspended in 15 ml of a protease inhibitor buffer (0.02 M potassium phosphate, pH 7.5, 0.1 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodiumdodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were then sonicated in an MSE Sonicator for 3 x 30 sec. on an ice-bath and centrifuged at 15,000 rpm

(Sorvall SS-34 rotor) for 10 min.

3 ml of supernatant was passed over an IgG-Sepharose® 4B column (Pharmacia AB, Uppsala, Sweden), as described by Hjelm et al, FEBS Lett. 28 (1972), that had been equilibrated with PBST buffer. The column was then washed with PBST and the adsorbed proteins were eluted with 3 ml glycine buffer (0.1 M glycine, 2% NaCl, pH 3.0). The eluate was dialyzed overnight against PBST and the concentration of protein A was determined by the ELISA-test as described in Routine Methods.

In order to determine whether any of the cultures contained a protein A - β-galactosidase fusion protein a modification of the test was performed. Before the addition of the protein A - alkaline phosphatase conjugate 100 µl of ONPG buffer was added to the wells of the microtiter plate and the colour change from colourless to yellow indicating β-galactosidase activity was determined visually. After three washes with PBST 50 µl of protein A - alkaline phosphatase conjugate was added and the test was continued exactly as described in Routine Methods. The results are shown in the following Table 1.

TABLE 1

Plasmid	β-galactosidase activity		Protein A concentration (µg/ml)	
	Total	Eluate	Total	Eluate
pSKS106	-	-	0	0
pSPA8	-	-	16	16
pSPA13	+	-	4	4
pSPA14	+	-	1	1

In the Table "Total" gives the value for cell lysate and "Eluate" gives the corresponding value after binding and elution from IgG-Sepharose®. As to the β-galactosidase activity a negative result represents no detectable colour change after incubation for 30 minutes at room temperature, whereas a positive result represents a clearly visible colour change after 5 minutes under the same conditions.

The test shows that the β-galactosidase from the control (pSKS106) does not bind to IgG coated wells in detectable amounts. In contrast, fusion proteins from cultures containing plasmids pSPA13 and pSPA14 bind to the wells and have enzymatic activity. The β-galactosidase activity is, however, not recovered after elution from the IgG-Sepharose® column with glycine-buffer (see Table 1). This is due to inactivation of the enzyme in glycine buffers under pH 4.

The ELISA-test shows that the protein A concentrations of the three protein A containing clones (pSPA8, pSPA13 and pSPA14) vary although the same protein A gene sequence with the same promoter was used. The two clones having the lac Z gene fused to the protein A gene (pSPA13 and pSPA14) contain less protein A than the pSPA8 clone. However, the protein A of the protein A containing clones (pSPA8, pSPA13 and pSPA14) binds to IgG-Sepharose® and can be eluted with high efficiency with glycine buffer of pH 3.0, as appears from Table 1, although the β-galactosidase of pSPA13 and pSPA14 is irreversibly inactivated.

In accordance with the above results desired enzymes may in this way be immobilized directly to an IgG affinity column from a cell crude lysate. Thus the specific affinity between IgG and protein A assures a one-step procedure giving a pure and immobilized enzyme.

VIII. Detection and quantitation of β-galactosidase activity from E. coli clones after immobilisation to IgG-Sepharose®

Cells carrying plasmids pSKS106, pSPA8, pSPA13 and pSPA14 were grown and lysed exactly as described in section VII. 10 ml of supernatants were mixed with 1 ml of sedimented IgG-Sepharose® 4B (Pharmacia AB, Uppsala, Sweden) that had been washed with PBST buffer. The mixtures were slowly inverted at 8°C for 1 h and the supernatants were collected. After 4 washes with 12 ml of PBST the supernatants of the last wash were collected. The Sepharose® was resuspended in 10 ml of PBST and aliquots were transferred to smaller tubes. The β-galactosidase activities were measured exactly as described in Routine Methods and the results are shown in the following Table 2.

TABLE 2

Plasmid	total β-galactosidase* activity (units/ml)	% activity in supernatant	% activity in wash	% activity immobilized
pSKS106	14	84.9	0.1	0.2
pSPA8	0	N.D.	N.D.	N.D.
pSPA13	0.4	3.0	0.3	71.6
pSPA14	0.1	6.0	0.0	78.4

* Values are calculated per ml of cell lysate

1 unit is defined as described under Routine Methods above.

N.D. = not detected

The β-galactosidase from cells containing plasmid pSKS106 (control) does not bind to IgG-Sepharose®, in accordance with the inability to bind to IgG coated wells (see step VII). In contrast, β-galactosidase from the cells containing plasmids pSPA13 and pSPA14 (protein A fusion proteins) binds efficiently, in fact more than 70% of the activity is immobilized.

From table 2 it appears that the fusion protein lacking the non-IgG-binding region of the protein A molecule (X in Fig. 2) as produced through plasmid pSPA13 gives 3-4 times more fusion product than the corresponding fusion product containing substantially the whole protein A gene, as obtained via plasmid pSPA14. It seems that in this particular case the "spacer" region X is less favourable.

IX. Elution of bound β-galactosidase-protein A-fusion protein from IgG-Sepharose®

Aliquots of IgG-Sepharose® suspension with bound fusion proteins from step VIII were transferred to columns. The fusion proteins were eluted from 50 µl of sedimented gel by adding 0,5 ml buffer containing purified protein A (Pharmacia, Uppsala, Sweden) at various concentrations at room temperature. The β-galactosidase activity of the eluates and the IgG-Sepharose® gels after elution were determined as described under Routine Methods above. The results are shown in Table 3, the values being expressed as percent of Sepharose®-bound β-galactosidase activity.

TABLE 3

Buffer	pSPA13		pSPA14	
	Immobilized after elution	eluate	Immobilized after elution	eluate
PBST	91	0	89	0
PBST + 0,5 mg protein A	63	35	62	30
PBST + 2 mg protein A	31	64	45	51

From the above results it appears that at least half the β-galactosidase activity may be eluted by this procedure.

EXAMPLE II

I. Construction of a shuttle plasmid containing a fusion between the genes encoding protein A and IGF-1

In the following steps A-D the construction of a plasmid pUN201, containing the protein A gene, without region X, fused to a synthetic gene encoding a modified human IGF-1 (human insulin-like growth factor type 1), is described.

A. Synthesis and cloning of the gene encoding human IGF-1

The oligomers shown in Fig. 13A were synthesised on an automatic DNA-synthesising machine developed by KabiGen AB, Sweden (Chow et al, Nucleic Acids Res. 9, 2807-2817) with N-protected nucleoside chlorophosphites as reagents (Elmblad et al, Nucleic Acids Res. 10, 3291-3301 (1982)). After purification and phosphorylation the oligomers were assembled into seven blocks as shown in Fig. 13B, which were then ligated as described in Fig. 13B.

In the last step, block A and block B in Fig. 13B were ligated giving the complete IGF-1 gene. This segment was digested with EcoRI and Hind III restriction enzymes and after purification inserted into plasmid pUC8 and transformed into E. coli JM83. The transformants were screened by colony hybridisation using A15 as probe and one of the positive clones, designated JM83/pKG3, was sequenced, confirming that the sequence matched the IGF-1 gene.

The synthesis of the IGF-1 gene is also described in the Swedish patent application 8303626-9, the disclosure of which is incorporated by reference herein. The DNA-sequence of the IGF-1 gene and the corresponding amino acid sequence appears from Fig. 11, except that a glycine residue (Gly) has been changed into an aspartic acid residue (Asp) as will be described in the following step B.

B. In vitro mutagenesis of the synthetic gene to encode a modified human IGF-1.

Oligonucleotide mediated in vitro mutagenesis was performed on the cloned synthetic IGF-1 gene, in order to change the part encoding the N-terminal amino acid residue of the mature protein. By changing this amino acid residue from a glycine to an aspartic acid residue the dipeptide aspartic acid-proline was formed. This allows for gene fusions encoding hybrid proteins that can be cleaved apart, before or after purification, by formic acid treatment which cleaves between aspartic acid and proline (Landon, Methods in Enzymology 47, 132-145, 1977). Thereby, mature IGF-1, lacking the N-terminal glycine, can be produced.

10 µg of plasmid pKG3 was cleaved with Eco RI and Hind III and a 0.22 kb fragment thereof was cut out from a 5% polyacrylamide gel after electrophoresis. The fragment was eluted and purified as described in the International patent application PCT/SE83/00297 (the disclosure of which is incorporated by reference herein). 50 ng of purified fragment was mixed with 200 ng of phage M13 mp9 and cleaved with Eco RI and Hind III in a total volume of 20 µl. After treatment with T4-ligase the DNA was used to transform E. coli JM83 and the cells were spread on AXI-plates. Cleavage, ligation and transformation were performed as described under Routine Methods. Phage purification from one white plaque was performed as described under Routine Methods. Using the universal primer (Bio-Labs, New England, USA) the phage insert was confirmed to be the 220 bp synthetic IGF-1 gene. This phage, designated mp9/IGF-1, was used for the following mutagenesis.

Two oligonucleotides were synthesized and purified exactly as described above and in the Swedish patent application No. 8303626-9. One primer oligonucleotide consisting of 24 bases (5'-GTGAATTCTATGGACCCCGAAACT-3') which was used for the mutagenesis and one probe (P) oligonucleotide consisting of 14 bases (5'-AATTCTATGGACCC-3') which was used to identify successfully mutagenized phage clones. The mismatches between the synthetic IFG-1 gene and the primer are shown in Fig. 9.

16 pmoles of mp9/IFG-1 and 80 pmoles of primer were mixed in a total volume of 80 µl containing 100 mM NaCl, 20 mM $MgCl_2$ and 40 mM TRIS-HCl, pH 7.5. The mixture was heated to 65°C for 3 minutes and allowed to cool to 23°C for 30 minutes. After transfer to an ice-bath, 190 µl of H_2O and 30 µl of a solution containing 100 mM $MgCl_2$, 50 mM DTT and 200 mM TRIS-HCl, pH 7.5, was added. 50 units of Klenow fragment (Boehringher-Mannheim, West-Germany) was added and after 10 minutes in an ice-bath the sample was brought to 23°C for 30 minutes. Another 50 units of Klenow fragment was added and after 60 minutes at 23°C the polymerase was heat inactivated at 65°C for 10 minutes. The sample was precipitated once with ethanol, followed by cleavage with Eco RI and Hind III according to Routine Methods above. The 0.22 kb fragment was cut out from a 5% polyacrylamide gel after electrophoresis and the fragment was eluted and purified as described in the above mentioned International patent application PCT/SE83/00297. 50 ng of purified fragment were mixed with 200 ng of phage M13 mp9 cleaved with Eco RI and Hind III in a total volume of 20 µl. After ligation and transformation to E. coli JM83, exactly as described above, white plaques were found in a background of blue plaques. 48 white plaques were

further analysed by hybridization with two synthetic probes as described by Winter et al (Nature 299, 21 October, 1982). The filters were hybridized at room temperature with ^{32}P-labelled oligonucleotides and washed at different temperatures. Using probe A2, 5'-ATGGGTCCCGAAAC-3', (Swedish patent application No. 8303626-9.), all clones except four show strong hybridization after wash at 44°C indicating that these clones contain the original IGF-1 gene. Using probe P, 5'-AATTCTATGGACCC-3', all four of the previously negative clones showed significant hybridization. One of the four phages, designated mp9/IGF-1.M3, was further sequenced using the universal primer as described above. This confirmed a successful mutagenesis as shown in Fig. 9.

200 ng of phage mp9/IGF-1.M3 and 200 ng of plasmid pUC8 were separately cleaved with Eco RI and Hind III. After T4-ligase treatment, in a total volume of 20 µl, the DNA was used to transform E. coli JM83 and the cells were plated out on AXI-plates. Cleavage, ligation and transformation were performed as described above under Routine Methods. Restriction analysis of a white colony revealed the expected plasmid, pUC8, containing a 0.22 kb Eco RI/Hind III insert. This plasmid was designated pKG11 and was used for the following steps.

C. Construction of shuttle plasmid pUN200 containing pKG11

1 µg of pKG11 from step B and 2 µg of pHV14, both digested with Hind III, were mixed and ligated in a total volume of 100 µl overnight at +14°C. After digestion with Eco RV, the DNA-mixture was transformed to E. coli HB101 and plated on LA-plates containing 50 µg ampicillin per milliliter. 52 single colonies were picked to LA plates containing 10 µg/ml of chloramphenicol and 50 µg/ml of ampicillin. After two days at 28°C one clone appeared and the plasmid in this clone was further characterized by restriction analysis. This revealed plasmid pUN200 schematically shown in Fig. 10. This plasmid, which contains the IGF-1 gene, can replicate both in E. coli and S. aureus.

D. Construction of shuttle plasmids pUN201 and pUN202

1 µg of pUN200 and 1 µg of plasmid pSPA16, both digested with Eco RI, were mixed and ligated in a total volume of 100 µl overnight at 14°C. The ligase was heat inactivated at 65°C for 10 minutes. After digestion with Eco RV to decrease the number of background clones containing pSPA16, the DNA mixture was transformed to E. coli HB101 and plated out on LA-plates containing 50 µg of ampicillin. Plasmids from 48 clones were analyzed by restriction mapping and 3 thereof were found to contain pUN200 with a 1,1 kb Eco RI insert from pSPA16, corresponding to the 5'-end of the protein A gene. The orientation of the insert in these three plasmids was further analyzed by cleavage with Hind III and

two were found to contain a predicted fusion between the genes encoding protein A and IGF-1. This plasmid was designated pUN201 (Fig. 10). The nucleotide sequence and the deduced amino acid sequence of this gene fusion are shown in Fig. 11. The predicted molecular weight of the mature hybrid protein is 38,701.

One of the three clones was found to contain a plasmid, designated pUN202, with opposite orientation of the protein A gene versus the IGF-1 gene (Fig. 10). This plasmid codes for a truncated protein A with a predicted molecular weight of 30,963 (Fig. 12).

II. Transformation of shuttle plasmids pUN201 and pUN202 to S. aureus SA113

10 µg of plasmids pUN201 and pUN202 from step ID above were used to transform protoplasts of S. aureus SA113 as described by Götz, F. et al, J. Bacteriol. 145, 74-81 (1981) and in the International patent application PCT/SE83/00297 (Step IIIA). Chloramphenicol resistant clones were found after 3 days at 37°C and these transformants were restreaked on TSA-plates (Trypticase Soy Agar) with chloramphenicol (10 µg/ml). One transformant of the respective plasmid (pUN201 and pUN202) was chosen for further analysis. Restriction mapping of the purified plasmids revealed that the intact plasmid had been introduced into the S. aureus SA113 host.

III. Quantification and localization of the protein A activity from clones carrying pUN201 and pUN202

E. coli cells carrying pUN200, pUN201 and pUN202 respectively (from Step IC and D) above and S. aureus cells carrying pUN201 or pUN202 were cultivated in 200 ml of liquid medium overnight. E. coli strains were grown in LB medium with ampicillin (50 µg/ml) and S. aureus strains in TSB (Trypticase Soy Broth) with chloramphenicol (10 µg/ml). The cells were pelleted by centrifugation at 6000 rpm with a Sorwall GSA-rotor for 10 minutes and the supernatant, designated medium, was saved. The cell pellet was washed in 10 ml of PBS + TWEEN and again centrifuged as above. This time the cell pellet was resuspended in 10 ml of a protease inhibitor buffer (0.02 M potassium phosphate, pH 7.5, 0.1 M NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodiumdodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were then sonicated in a MSE sonicator for 4 x 40 sec. on an ice-bath and centrifuged at 15,000 rpm (Sorvall SS-34 rotor) for 10 min. The supernatant, designated cell extract, was collected and the ELISA-test described under Routine Methods was performed to determine the amount of protein A in the samples. The results are shown in the following Table 4.

Table 4

Amount of protein A per ml of sonicated cell culture determined by the ELISA-test. Zero-values correspond to less than 0.1 µg/ml.

Host (Plasmid)	Cell extract (µg/ml)	Medium (µg/ml)
E. coli HB101 (pUN200)	0	0
E. coli HB101 (pUN201)	2	0
E. coli HB101 (pUN202)	2	0
S. aureus SA113 (pUN201)	0,2	5
S. aureus SA113 (pUN202)	0	5
E. coli HB 101	0	0
S. aureus SA113	0	0

Table 4 shows that in both E. coli and S. aureus the amount of protein A produced is not influenced by the orientation of the fragment containing the protein A gene (plasmid pUN201 versus pUN202). Thus, the protein A IGF-1 hybrid protein encoded by pUN201 is produced at approximately the same level as the truncated protein A encoded by pUN202. Both proteins are, as expected, found in the cell extract of E. coli and in the medium of S. aureus.

IV. Purification of IGF-1 by IgG-affinity chromatography and formic acid treatment

The media of S. aureus SA113 carrying pUN201 and pUN202 respectively, from Step III, were each passed over an IgG-Sepharose® 4B column (Pharmacia AB, Uppsala, Sweden) (Hjelm et al, FEBS Lett. 28, 73-76 (1972)) that had been equilibrated with a sodium acetate buffer (0.1 M sodium acetate, 2% NaCl, pH 5.5). The column was then washed with the same buffer as above and the adsorbed protein A eluted with a glycine buffer (0.1 M glycine, 2% NaCl, pH 3.0). The eluted fraction was dialyzed against distilled water and thereafter lyophilized in two aliquots. The protein pellet of one of the aliquots was analysed on a 13% SDS-polyacrylamide gel at 100 V for 12 hours. The gel was stained with amidoblack (0.1%, in 45% methanol, 10% acetic acid). This revealed a major protein with the molecular weight of 38,701 for pUN210 and 30,963 for pUN202. This is in accordance with the predicted sizes deduced from the DNA sequences (see Step ID above).

The second aliquot was resuspended in 0.5 ml of 70% formic acid and

further incubated at 37°C for 2 days. This process cleaves proteins at the dipeptide sequence aspartic acid-proline. The predicted degradation products from the hybrid protein encoded by pUN201 were, apart from the IGF-1 moiety lacking the N-terminal glycine, five oligopeptides of the molecular weights of 6800, 6600, 6600, 6600 and 600. SDS-polyacrylamide electrophoresis, as described above, also confirms that the major protein bands are shifted from approximately 38000 to several bands around 7000.

The formic acid treated proteins were lyophilized and resuspended in distilled water. The sample from pUN201 was passed over an IgG-Sepharose® 4B column as described above. The flow through and the eluted material (with the glycine buffer) were saved for further analysis.

V. Analysis of the protein products by radio receptor assay (RRA)

The radio receptor assay (RRA) was performed according to Hall et al, J. Clin. Endocrinol. Metab. 48, 271-278 (1974) using a particulate fraction of human placental membrane as matrix. The standard used in the assay consisted of pooled normal human serum having a potency of 1 unit (U) of IGF-1 per ml. The lower limit of the assay was 10 mU per ml. The peptide used for labelling was purified from Cohn fraction IV to a specific activity of 500 U/mg protein (according to RRA). Labelling of the peptide was performed according to Thorell et al. Biochem. Biophys. Acta 251, 363-369 (1971). Purification of the tracer was done on a carboxymethyl cellulose column using an elution gradient of 0.1 M NH_4OAc from pH 4.0 to pH 6.8. The specific activity of the tracer was approximately 20 µCi/µg. The assay was performed as follows:

The standard or unknown sample (100 µl) was incubated together with 100 µl of placental membrane and 100 µl of labelled IGF-1 overnight at +4°C. After centrifugation the pellet was washed once and counted in a gamma counter. The sample potency was calculated using an "in house" computer program.

Samples before and after the formic acid treatment, from Step IV above, were analyzed by the RRA-test and the results are shown in Table 5 below.

Table 5

Radio receptor analysis (RRA) for IGF-1 activity in growth medium from S. aureus SA113 (pUN202) and S. aureus SA113 (pUN201) after isolation and purification by IgG affinity chromatography. Ze o corresponds to less than 1 U/l medium.

Plasmid	Treatment	Activity/l (U/l)
pUN202	Before treatment with formic acid	0
	After treatment with formic acid	0
pUN201	Before treatment with formic acid	0
	After treatment with formic acid	143
	Flow through, *	106
	Eluate, *	19

* A formic acid treated sample was passed over an IgG-Sepharose® column and the activity was measured for bound (eluate) and not bound (flow through) IGF-1.

From Table 5 it appears that the hybrid protein encoded by pUN201 has no detectable IGF-1 activity. Treatment with formic acid yields IGF-1 activity, and most of this activity does not bind to the IgG affinity column indicating a successful cleavage between the protein A and the IGF-1 moiety.

While embodiments of the invention have been presented above, the invention is, of course, not restricted thereto, but many variations and modifications are possible without departing from the scope thereof as defined by the subsequent claims.

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 14, line 2-6 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depositary institution (including postal code and country) [5]

Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany

Date of deposit [6]	Accession Number [7]
July 12, 1982	DSM 2434

B. ADDITIONAL INDICATIONS [8] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [9] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [10] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [11] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer) May Rydius

☒ The date of receipt (from the applicant) by the International Bureau [12]

was REC'D 24 FEB 1984 WIPO PCT

(Authorized Officer) Jordan FRANKLIN

BUREAU OMPI INTERNATIONAL WIPO

INTERNATIONAL FORM

Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581
S-75123 Uppsala
Schweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: E. coli SPA11	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 2434

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☒ a scientific description

☒ a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on July 12, 1982 (Date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN

Address: Grisebachstrasse 8
D-3400 Göttingen

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

D. Claus

Date: July 13, 1982

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

Form DSM-BP/4 (sole page) 0582

Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581

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Schweden

VIABILITY STATEMENT
issued pursuant to rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the bottom of this page

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Martin Lindberg Address: Department of Microbiology Biomedical Center Box 581 S-75123 Uppsala, Schweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY DSM 2434 Date of the deposit or of the transfer: [1] July 12, 1982

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on [2]
On that date, the said microorganism was

- [x] [3] viable
- [ ] [3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN Address: Grisebachstr. 8 D-3400 Göttingen	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: July 13, 1982

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

[4] Fill in if the information has been requested and if the results of the tests were negative.

Form DSMZ-BP 9 (sole sheet) 0582

BUREAU

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 14, line 2-6 of the description [5]

A. IDENTIFICATION OF DEPOSIT [6]

Further deposits are identified on an additional sheet ☐ [6]

Name of depositary institution [6]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depositary institution (including postal code and country) [6]

Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany

Date of deposit [6]	Accession Number [6]
August 15, 1983	DSM 2706

B. ADDITIONAL INDICATIONS [7] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [8] (If the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [9] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [9] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

May Rydius

(Authorized Officer) May Rydius

☒ The date of receipt (from the applicant) ~~by the~~ International Bureau [10]

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J Franklin

(Authorized Officer) Jordan FRANKLIN

BUREAU OMPI WIPO INTER...

Martin Lindberg
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Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: KL117 (pSPA16)	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 2706

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on August 15,1983 (Date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN

Address: Grisebachstrasse 8
D-3400 Göttingen

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

D. Claus

Date: August 23,1983

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

VIABILITY STATEMENT
issued pursuant to rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the bottom of this page

Martin Lindberg
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Box 581

S-751 23 Uppsala
Sweden

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Martin Lindberg,Dept.of Microbiology, Biomedical Center Address: Box 581 S-751 23 Uppsala Sweden	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY DSM 2706 Date of the deposit or of the transfer: [1] August 15,1983

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on Aug.22,1983 . [2]
On that date, the said microorganism was

- [x] [3] viable
- [ ] [3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN Address: Grisebachstr. 8 D-3400 Göttingen	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: August 23,1983

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

[4] Fill in if the information has been requested and if the results of the tests were negative.

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 22, line 37 - page 23, line 1 of the description

A. IDENTIFICATION OF DEPOSIT

Further deposits are identified on an additional sheet ☐

Name of depositary institution

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depositary institution (including postal code and country)

Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany

Date of deposit	Accession Number
February 4, 1983	DSM 2591

B. ADDITIONAL INDICATIONS (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer) May Rydius

☒ The date of receipt (from the applicant) by the International Bureau

was RECD 24 FEB 1984 WIPO PCT

(Authorized Officer) Jordan FRANKLIN

BUREAU OMPI

Form PCT/RO/134 (January 1981)

INTERNATIONAL FORM

Dr.Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581

S-75123 Uppsala
Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: SPA 23	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 2591

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on February 4, 1983 (Date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN

Address: Grisebachstrasse 8
D-3400 Göttingen

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

D. Claus

Date: February 17, 1983

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

VIABILITY STATEMENT
issued pursuant to rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the bottom of this page

Dr.Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581
S-75123 Uppsala
Sweden

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Dr.M.Lindberg Department of Microbiology Biomedical Center Address: Box 581 S-75123 Uppsala	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY DSM 2591 Date of the deposit or of the transfer: [1] February 4,1983

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on Feb.8,1983. [2]
On that date, the said microorganism was

- [x] [3] viable
- [ ] [3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN Address: Grisebachstr. 8 D-3400 Göttingen	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): D. Claus Date: February 17,1983

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(a)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

[4] Fill in if the information has been requested and if the results of the tests were negative.

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 23, line 16-18 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

Deutsche Sammlung von Mikroorganismen (DSM)

Address of depositary institution (including postal code and country) [5]

Grisebachstrasse 8, D-3400 Göttingen, Federal Republic of Germany

Date of deposit [6]	Accession Number [7]
February 4, 1983	DSM 2592

B. ADDITIONAL INDICATIONS [8] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [9] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [10] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [11] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the international application when filed (to be checked by the receiving Office)

(Authorized Officer) May Rydius

☒ The date of receipt (from the applicant) by the International Bureau [12] was

REC'D 2 4 FEB 1984
WIPO - PCT

(Authorized Officer) Jordan FRANKLIN

BUREAU OMPI INTERNATIONAL WIPO

Form PCT/RO/134 (January 1981)

INTERNATIONAL FORM

Dr.Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581

S-75123 Uppsala
Sweden

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

I. IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR: SPA 24	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: DSM 2592

II. SCIENTIFIC DESCRIPTION AND/OR TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

[X] a scientific description

[X] a proposed taxonomic designation

(Mark with a cross where applicable)

III. RECEIPT AND ACCEPTANCE

This International Depositary Authority accepts the microorganism identified under I above, which was received by it on February 4,1983 (Date of the original deposit)[1]

IV. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN

Address: Grisebachstrasse 8
D-3400 Göttingen

Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s):

D. Claus

Date: February 17, 1983

[1] Where Rule 6.4(d) applies, such date is the date on which the status of international depositary authority was acquired; where a deposit made outside the Budapest Treaty after the acquisition of the status of international depositary authority is converted into a deposit under the Budapest Treaty, such date is the date on which the microorganism was received by the international depositary authority.

VIABILITY STATEMENT
issued pursuant to rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the bottom of this page

Dr.Martin Lindberg
Department of Microbiology
Biomedical Center
Box 581
S-75123 Uppsala
Sweden

I. DEPOSITOR	II. IDENTIFICATION OF THE MICROORGANISM
Name: Dr.M.Lindberg Department of Microbiology Biomedical Center Address: Box 581 S-75123 Uppsala	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY DSM 2592 Date of the deposit or of the transfer: [1] February 4,1983

III. VIABILITY STATEMENT

The viability of the microorganism identified under II above was tested on Feb.8,1983. [2]
On that date, the said microorganism was

- [X] [3] viable
- [ ] [3] no longer viable

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]

V. INTERNATIONAL DEPOSITARY AUTHORITY

Name: DEUTSCHE SAMMLUNG VON MIKROORGANISMEN Address: Grisebachstr. 8 D-3400 Göttingen	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s): Date: February 17,1983

[1] Indicate the date of original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).

[2] In the cases referred to in Rule 10.2(c)(ii) and (iii), refer to the most recent viability test.

[3] Mark with a cross the applicable box.

[4] Fill in if the information has been requested and if the results of the tests were negative.

CLAIMS

1. A method of producing and selectively isolating a desired protein or polypeptide or derivative thereof, characterized by the steps of

constructing a recombinant vector comprising a DNA sequence coding for said desired protein or polypeptide operatively linked to a DNA sequence coding for protein A or an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, such that said DNA sequences together code for an IgG-binding fusion product between said desired protein or polypeptide and said protein A, active polypeptide fragment thereof or macromolecule;

transforming a compatible host with said recombinant vector such that the combined DNA sequences coding for said fusion protein or polypeptide can be expressed by the host, and culturing the transformed host in a suitable growth medium to produce said fusion protein or polypeptide;

selectively isolating said fusion protein or polypeptide by adsorption to an IgG-supporting carrier material, and

optionally desorbing said fusion protein or polypeptide from said IgG-supporting carrier.

2. A method according to claim 1, characterized in that said isolation of the fusion protein or polypeptide is performed on a lysate of the cultured host cells or the growth medium.

3. A method according to claim 1 or 2, characterized in that the fusion protein or polypeptide coded for by said combined DNA-sequence comprises a unique cleavage site, which is not present in the desired protein and preferably not in the protein A part, between said protein A part and said desired protein or polypeptide part, and that said desired protein or polypeptide part is cleaved off from the rest of the fusion protein or polypeptide either while the latter is adsorbed to the IgG-supporting carrier or after desorption thereof from the carrier.

4. A method according to claim 3, characterized in that said unique cleavage site is an amino acid or amino acid sequence susceptible to cleavage by a cleaving agent selected from the group consisting of proteases, hydroxylamine, cyanogen bromide and formic acid.

5. A method according to any one of claims 1-4, characterized in that said recombinant vector is constructed by providing an expression vector comprising a

functional DNA sequence coding for protein A, an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, and a multilinker sequence located before any stop codon of the protein A coding sequence; and inserting a DNA sequence coding for said desired protein or polypeptide into an appropriate restriction site of said multilinker sequence, and optionally inserting a DNA sequence coding for said unique cleavage site between the DNA sequence coding for protein A and the DNA sequence coding for the desired protein or polypeptide, said cleavage site coding sequence preferably being provided in the expression vector or in the junction end of the desired protein or polypeptide coding DNA sequence before the insertion thereof into the expression vector.

6. A method according to any one of claims 1-5, characterized in that said desorption of the fusion protein or polypeptide from the IgG-supporting carrier is effected by subjecting the carrier to low pH conditions, high salt concentrations, chaotrophic ions or to competitive protein A elution with an excess of soluble protein A or IgG or fragments thereof.

7. A recombinant vector, characterized in that it comprises a DNA sequence coding for protein A or an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, operatively linked to a DNA sequence coding for a desired protein or polypeptide, such that the combined sequences together code for a fusion product between said protein A, active polypeptide fragment thereof or macromolecule and said desired protein or polypeptide, said fusion product having IgG-binding activity.

8. A recombinant vector according to claim 7, characterized in that said protein A coding DNA sequence extends from the 5'-end of the combined DNA sequence coding for said fusion protein or polypeptide, said protein A coding sequence preferably comprising the promoter and signal sequence of the structural protein A coding gene.

9. A recombinant vector according to claim 7 or 8, characterized in that it in the junction between said combined DNA sequences comprises a DNA sequence coding for a unique cleavable site, which is not present in said desired protein or polypeptide and preferably not in said protein A part and which may be cleaved by a cleaving agent.

10. A recombinant vector according to claim 9, characterized in that said unique cleavable site is an amino acid or amino acid sequence susceptible to cleavage by a cleaving agent selected from the group consisting of proteases, cyanogen bromide, hydroxylamine and formic acid.

11. A recombinant vector according to any one of claims 7-10, characterized in that it is a plasmid.

12. A method of preparing the recombinant vector of any one of claims 7-11, characterized in that it comprises the steps of providing an expression vector comprising a functional DNA sequence coding for protein A, an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, and a multilinker sequence located before any stop codon of the protein A coding sequence, inserting a DNA sequence coding for said desired protein or polypeptide into an appropriate restriction site of said multilinker sequence, and optionally inserting a DNA sequence coding for a unique cleavage site, which is not present in the desired protein or polypeptide and preferably not in the protein A part, between the DNA sequence coding for said protein A and the DNA sequence coding for the desired protein or polypeptide, said cleavage site coding sequence preferably being provided in the expression vector or in the junction end of the desired protein or polypeptide coding DNA sequence before the insertion thereof into the expression vector.

13. An expression vector, characterized in that it comprises a functional DNA sequence coding for protein A, an active polypeptide fragment thereof, or any other macromolecule capable of binding to the constant regions of immunoglobulins, and a multilinker sequence located before any stop codon of the protein A coding sequence.

14. A host organism transformed by the recombinant vector of any one of claims 7-12, preferably a strain of Escherichia, Bacillus or Staphylococcus.

15. A fusion protein or polypeptide, characterized in that it is produced according to any one of claims 1, 2, 5 and 6.

16. A fusion protein or polypeptide according to claim 15, characterized in that it is bound to an IgG-supporting carrier material.

17. A method of producing a preparation of a desired protein or polypeptide or derivative thereof, from which the latter can be readily isolated, characte-

rized by the steps of

constructing a recombinant vector comprising a DNA sequence coding for said desired protein or polypeptide operatively linked to a DNA sequence coding for protein A or an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, such that said DNA sequences together code for an IgG-binding fusion product between said desired protein or polypeptide and said protein A, active polypeptide fragment thereof or macromolecule; and

transforming a compatible host with said recombinant vector such that the combined DNA sequences coding for said fusion protein or polypeptide can be expressed by the host, and culturing the transformed host in a suitable growth medium to produce said fusion protein or polypeptide.

AMENDED CLAIMS

[received by the International Bureau on 09 July 1984 (09.07.84); original claims 1-17 have been amended]

1. A method of producing and selectively isolating a desired protein or polypeptide or derivative thereof, characterized by the steps of

constructing a recombinant vector comprising a DNA sequence coding for said desired protein or polypeptide operatively linked to a DNA sequence coding for protein A or an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, such that said DNA sequences together code for an IgG-binding fusion product between said desired protein or polypeptide and said protein A, active polypeptide fragment thereof or macromolecule;

transforming a compatible host with said recombinant vector such that the combined DNA sequences coding for said fusion protein or polypeptide can be expressed by the host, and culturing the transformed host in a suitable growth medium to produce said fusion protein or polypeptide;

selectively isolating said fusion protein or polypeptide by adsorption to IgG or Fc-part thereof immobilized on a carrier material, and

optionally desorbing said fusion protein or polypeptide from said IgG-supporting carrier.

2. A method according to claim 1, characterized in that said isolation of the fusion protein or polypeptide is performed on a lysate of the cultured host cells or the growth medium.

3. A method according to claim 1 or 2, characterized in that the fusion protein or polypeptide coded for by said combined DNA-sequence comprises a unique cleavage site, which is not present in the desired protein and preferably not in the protein A part, between said protein A part and said desired protein or polypeptide part, and that said desired protein or polypeptide part is cleaved off from the rest of the fusion protein or polypeptide either while the latter is adsorbed to the carrier or after desorption thereof from the carrier.

4. A method according to claim 3, characterized in that said unique cleavage site is an amino acid or amino acid sequence susceptible to cleavage by a cleaving agent selected from the group consisting of proteases, hydroxylamine, cyanogen bromide and formic acid.

5. A method according to any one of claims 1-4, characterized in that said recombinant vector is constructed by providing an expression vector comprising a

functional DNA sequence coding for protein A, an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, and a multilinker sequence located before any stop codon of the protein A coding sequence; and inserting a DNA sequence coding for said desired protein or polypeptide into an appropriate restriction site of said multilinker sequence, and optionally inserting a DNA sequence coding for said unique cleavage site between the DNA sequence coding for protein A and the DNA sequence coding for the desired protein or polypeptide, said cleavage site coding sequence preferably being provided in the expression vector or in the junction end of the desired protein or polypeptide coding DNA sequence before the insertion thereof into the expression vector.

6. A method according to any one of claims 1-5, characterized in that said desorption of the fusion protein or polypeptide from the carrier is effected by subjecting the carrier to low pH conditions, high salt concentrations, chaotrophic ions or to competitive protein A elution with an excess of soluble protein A or IgG or fragments thereof.

7. A recombinant vector, characterized in that it comprises a DNA sequence coding for protein A or an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, operatively linked to a DNA sequence coding for a desired protein or polypeptide, such that the combined sequences together code for a fusion product between said protein A, active polypeptide fragment thereof or macromolecule and said desired protein or polypeptide, said fusion product having IgG-binding activity.

8. A recombinant vector according to claim 7, characterized in that said protein A coding DNA sequence extends from the 5'-end of the combined DNA sequence coding for said fusion protein or polypeptide, said protein A coding sequence preferably comprising the promoter and signal sequence of the structural protein A coding gene.

9. A recombinant vector according to claim 7 or 8, characterized in that it in the junction between said combined DNA sequences comprises a DNA sequence coding for a unique cleavable site, which is not present in said desired protein or polypeptide and preferably not in said protein A part and which may be cleaved by a cleaving agent.

10. A recombinant vector according to claim 9, characterized in that said unique cleavable site is an amino acid or amino acid sequence susceptible to cleavage by a cleaving agent selected from the group consisting of proteases, cyanogen bromide, hydroxylamine and formic acid.

11. A recombinant vector according to any one of claims 7-10, characterized in that it is a plasmid.

12. A method of preparing the recombinant vector of any one of claims 7-11, characterized in that it comprises the steps of providing an expression vector comprising a functional DNA sequence coding for protein A, an active polypeptide fragment thereof or any other macromolecule capable of binding to the constant regions of immunoglobulins, and a multilinker sequence located before any stop codon of the protein A coding sequence, inserting a DNA sequence coding for said desired protein or polypeptide into an appropriate restriction site of said multilinker sequence, and optionally inserting a DNA sequence coding for a unique cleavage site, which is not present in the desired protein or polypeptide and preferably not in the protein A part, between the DNA sequence coding for said protein A and the DNA sequence coding for the desired protein or polypeptide, said cleavage site coding sequence preferably being provided in the expression vector or in the junction end of the desired protein or polypeptide coding DNA sequence before the insertion thereof into the expression vector.

13. An expression vector, characterized in that it comprises a functional DNA sequence coding for protein A, an active polypeptide fragment thereof, or any other macromolecule capable of binding to the constant regions of immunoglobulins, and a multilinker sequence located before any stop codon of the protein A coding sequence.

14. A host organism transformed by the recombinant vector of any one of claims 7-12, preferably a strain of Escherichia, Bacillus or Staphylococcus.

15. A fusion protein or polypeptide, characterized in that it is produced according to any one of claims 1, 2, 5 and 6.

16. A fusion protein or polypeptide according to claim 15, characterized in that it is bound to a carrier material supporting IgG or Fc-part thereof.

17. A method of producing a preparation of a desired protein or polypeptide or derivative thereof, from which the latter can be readily isolated, characte-

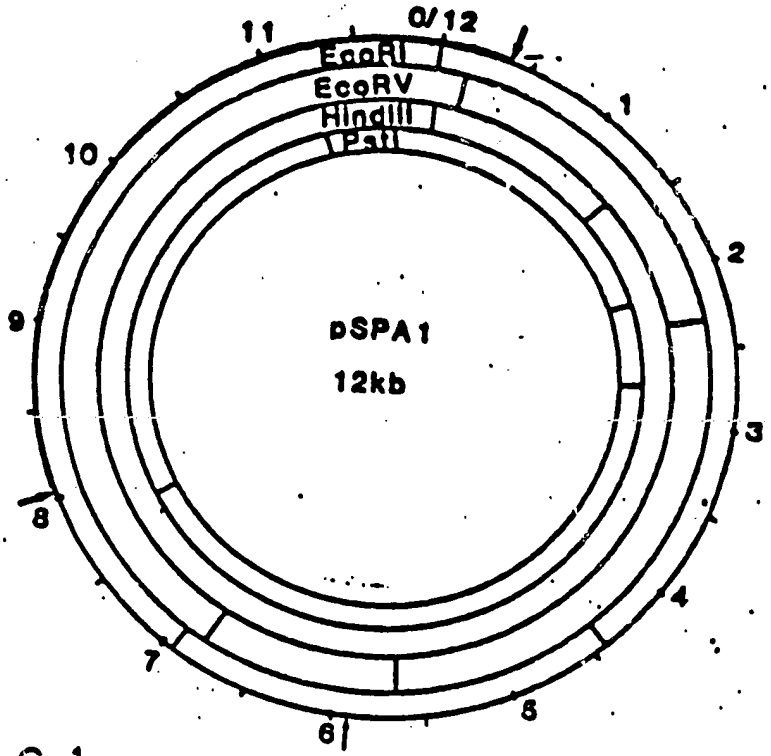


FIG.1

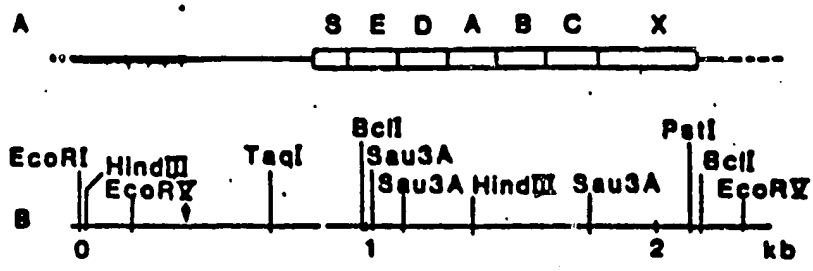


FIG. 2

```
    1069          1081          1093          1105          1117
      |             |             |  Sau3A      |             |
A.    AACGGCTTCATC CAAAGCCTTAAA GACGATCCTTCG GTGAGCAAAGAA ATTTTAGCAGAA
      AsnGlyPheIle GlnSerLeuLys AspAspProSer ValSerLysGlu IleLeuAlaGlu

    1513          1525          1537          1549          1561
      |             |             |  PstI       |             |
B.    GCAAACGGCACT ACTGCTGACAAA ATTGCTGCAGAT AACAAATTGGCT GATAAAAACATG
      AlaAsnGlyThr ThrAlaAspLys IleAlaAlaAsp AsnLysLeuAla AspLysAsnMet
```

FIG. 3

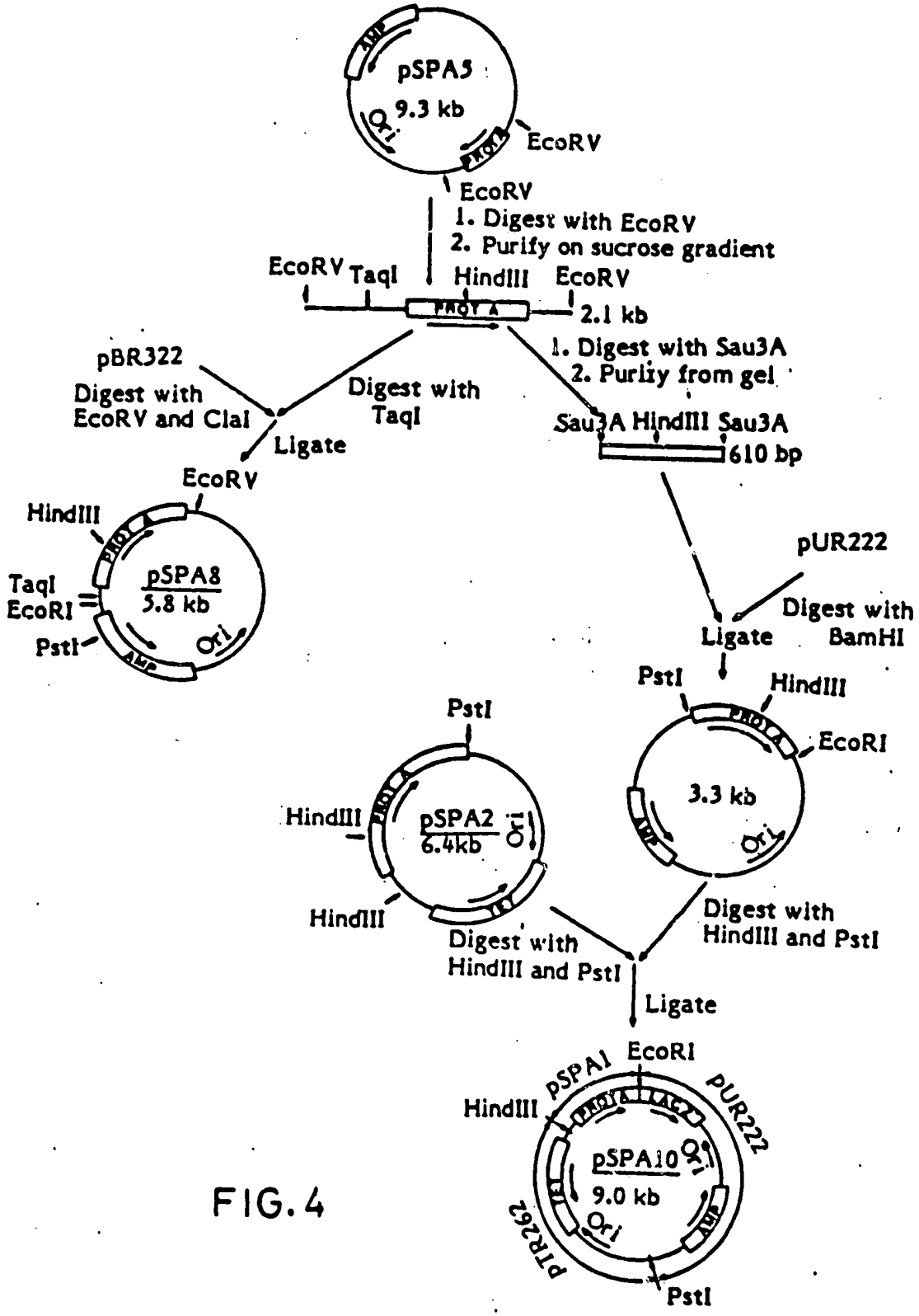


FIG. 4

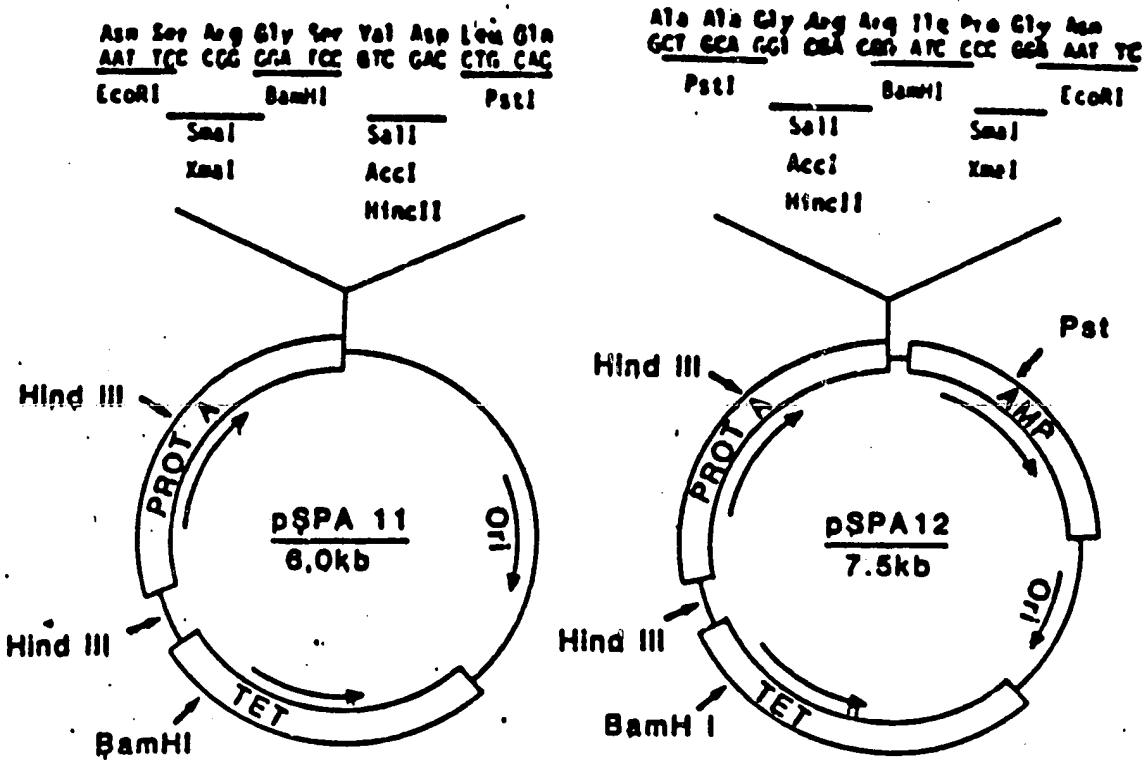


FIG. 5

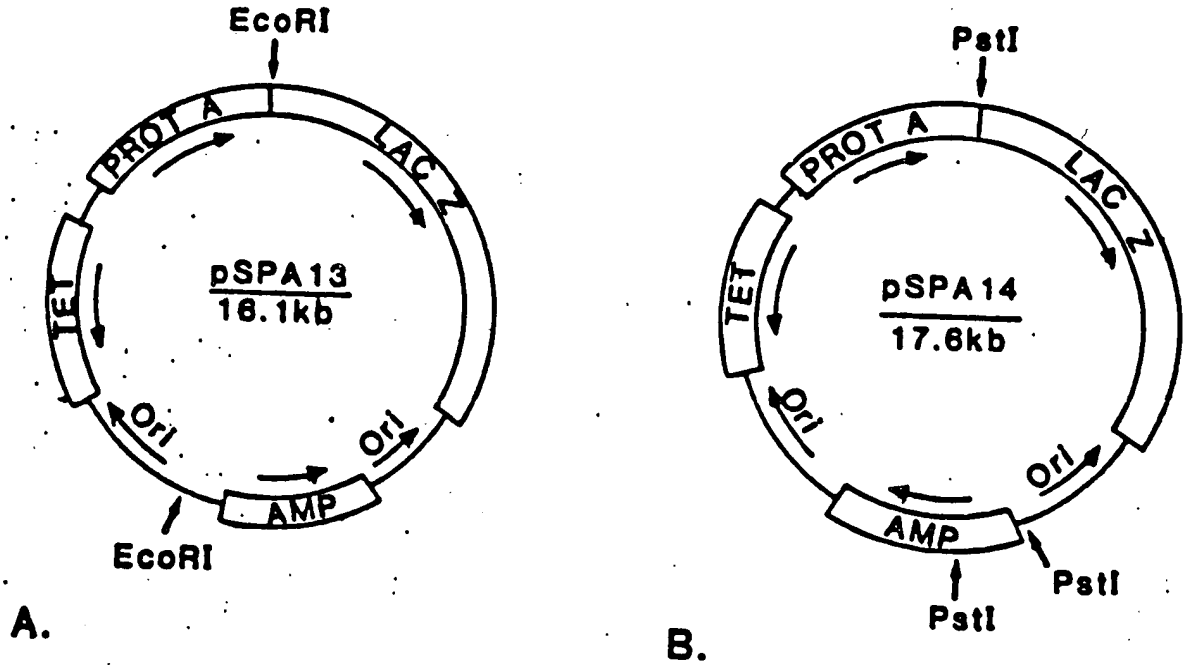


FIG. 6

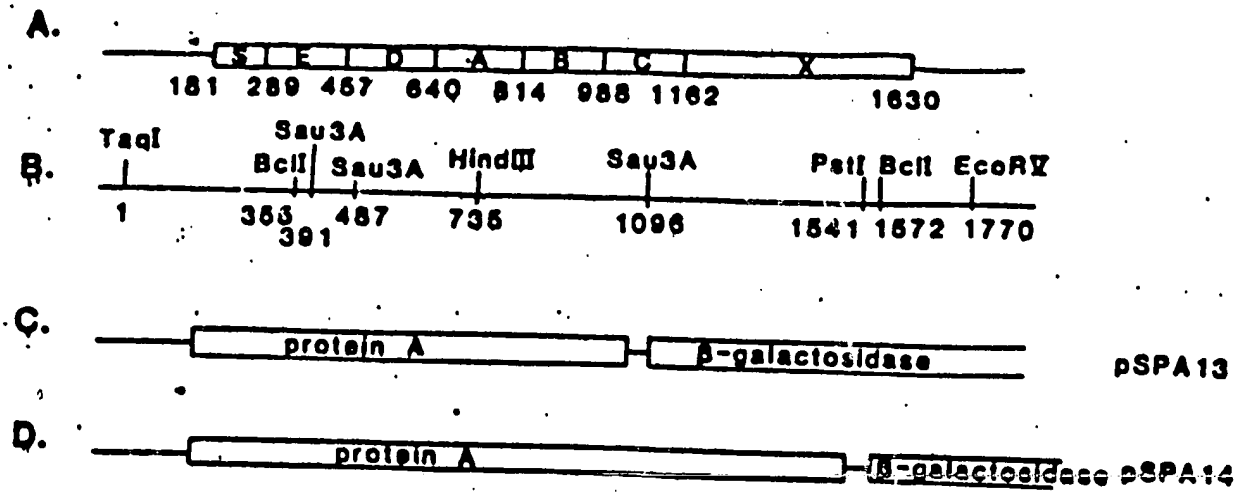


FIG. 7

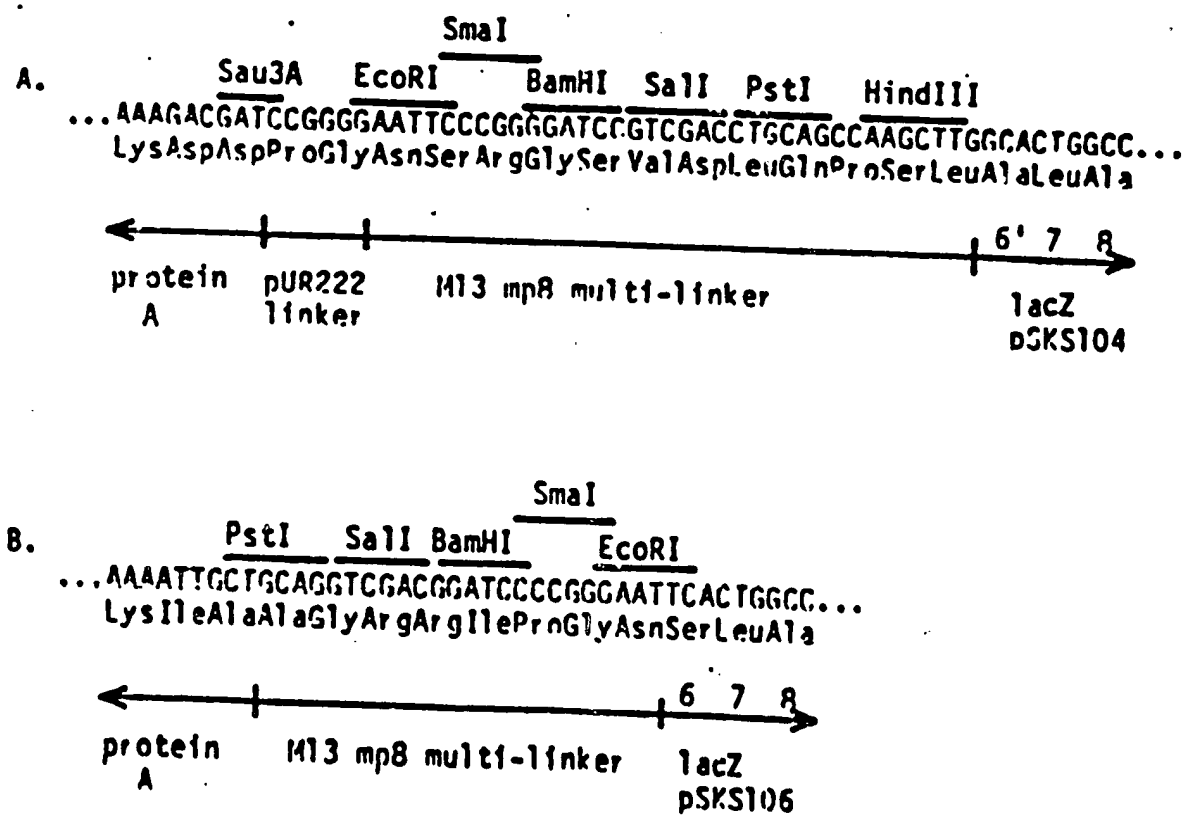


FIG. 8

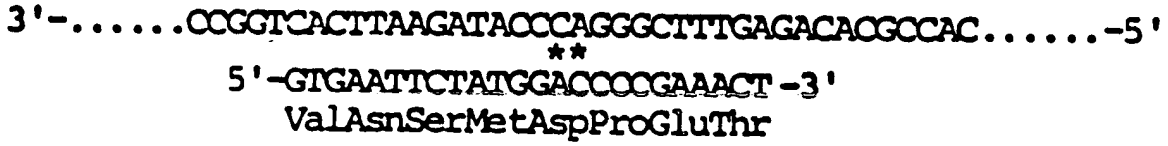


FIG. 9

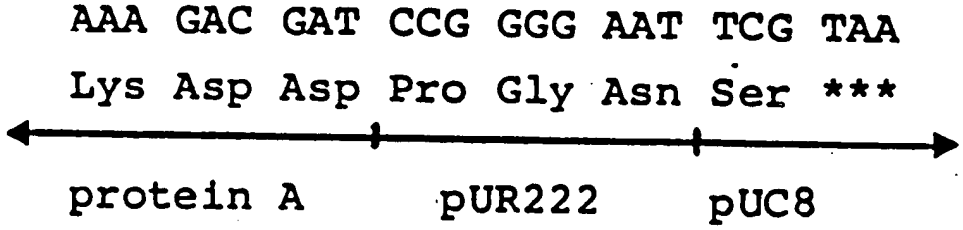


FIG. 12

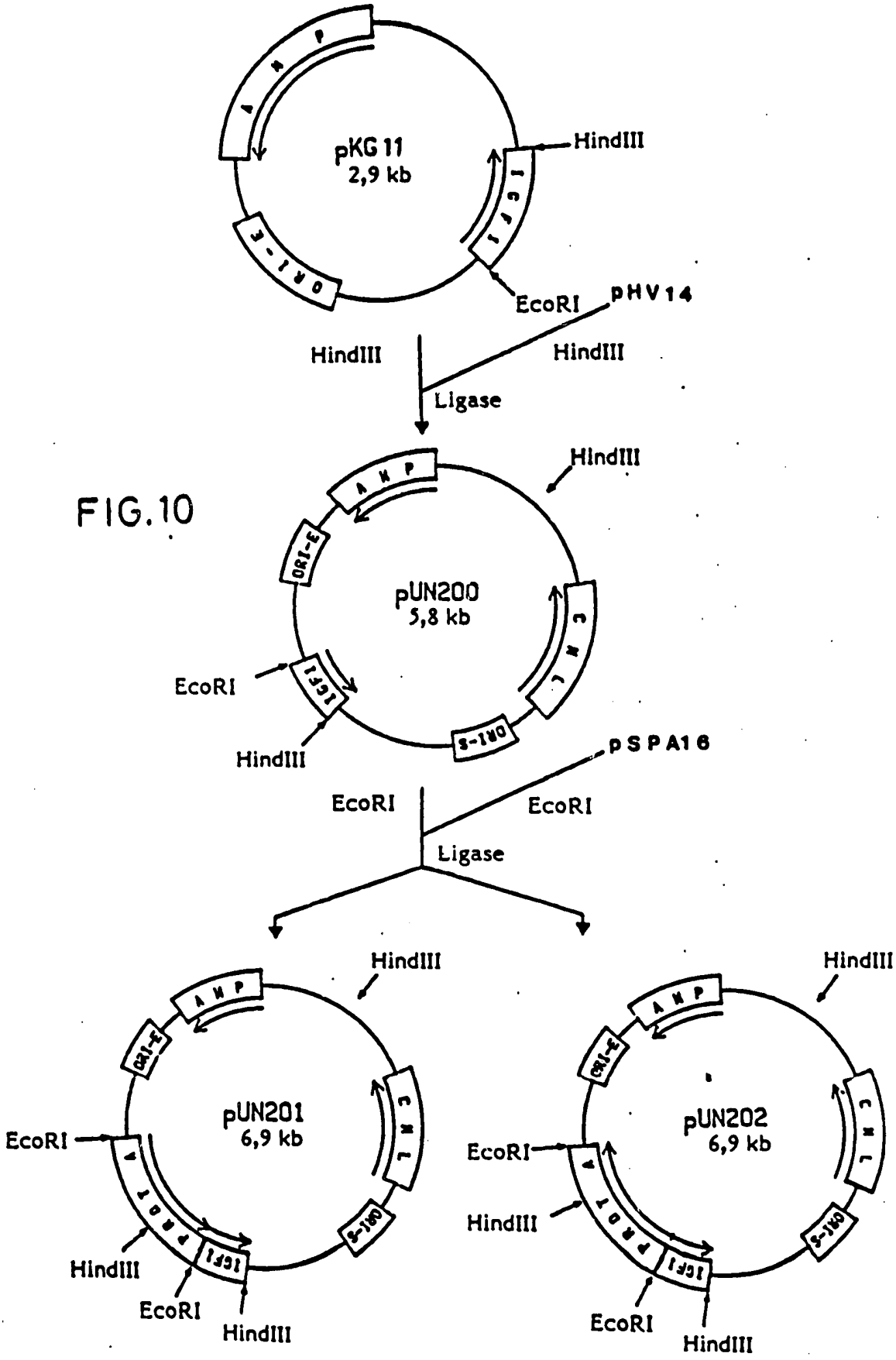
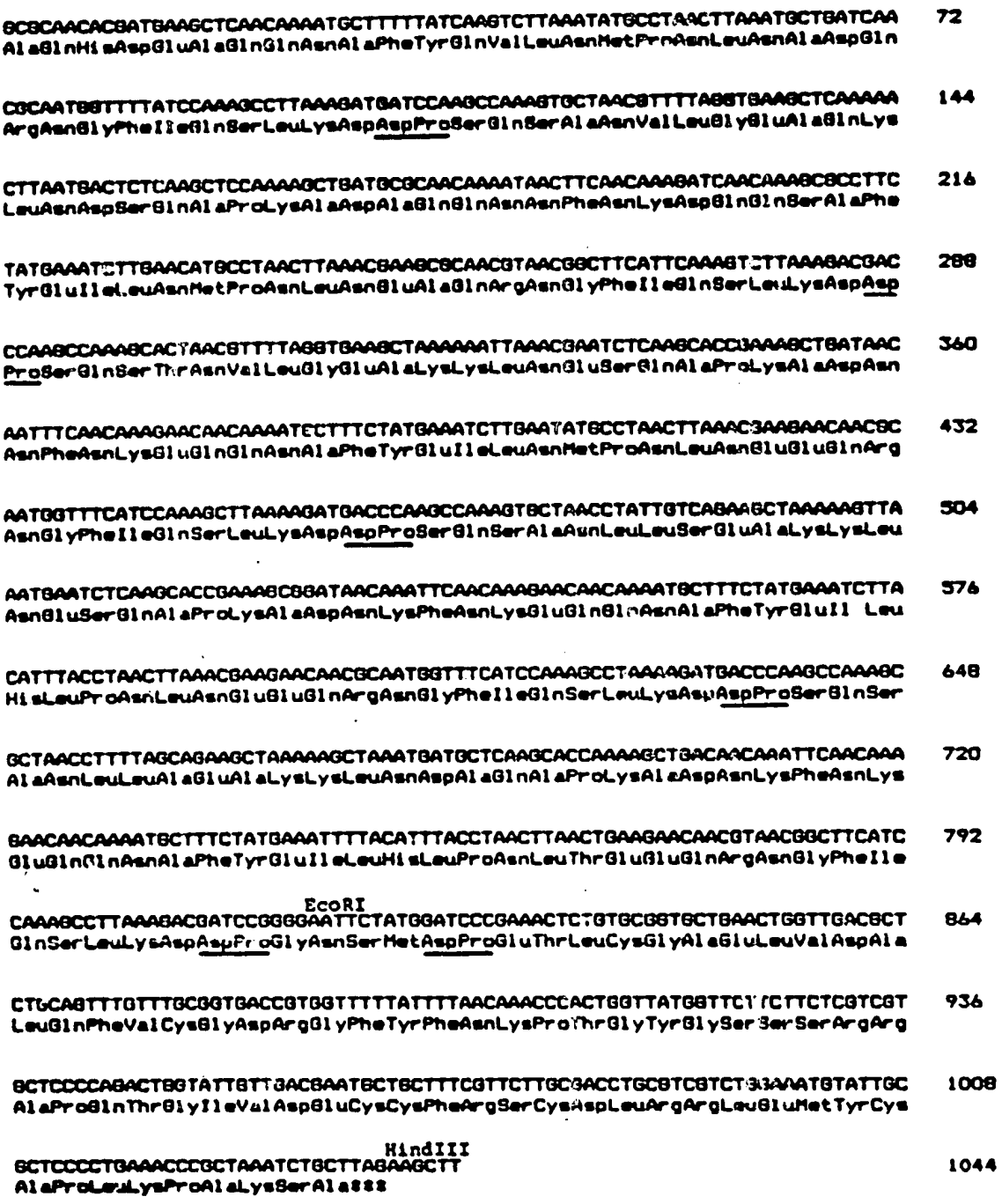


FIG.10

FIG. 11



```
5'  A1          A2              A3              A4              A5
    GGAATTCT ATGGGTCCCGAAAC TCTGTGCGGTGCTG AACTGGTTGACGCT CTGCAGTTTGTTTG

3'  CCTTAAGATACCCA GGGCTTTGAGACAC GCCACGACTTGACC AACTGCGAGACGTC
        B17             B16             B15             B14

                A6              A7              A8              A9
          CGGTGACCGTGGTT TTTATTTTAACAAA CCCACTGGTTATGG TTCTTCTTCTCGTC

AAACAAACGCCACT GGCACCAAAAATAA AATTGTTTGGGTGA CCAATACCAAGAAG AAGAGCAGCACGAG
    B13             B12             B11             B10             B9

    A10             A11             A12             A13
GTGCTCCCCAGACT GGTATTGTTGACGA ATGCTGCTTTCGTT CTTGCGACCTGCGT

      GGGTCTGACCATAA CAACTGCTTACGAC GAAAGCAAGAACGC TGGACGCAGCAGAC
          B8              B7              B6              B5

    A14             A15             A16             A17
CGTCTGGAAATGTA TTGCGCTCCCCTGA AACCCGCTAAATCT GCTTAGAAGCTTGG  3'

    CTTTACATAACGCG AGGGGACTTTGGGC GATTTAGACGAATC TTCGAACC  5'
          B4              B3              B2              B1
```

FIG. 13A

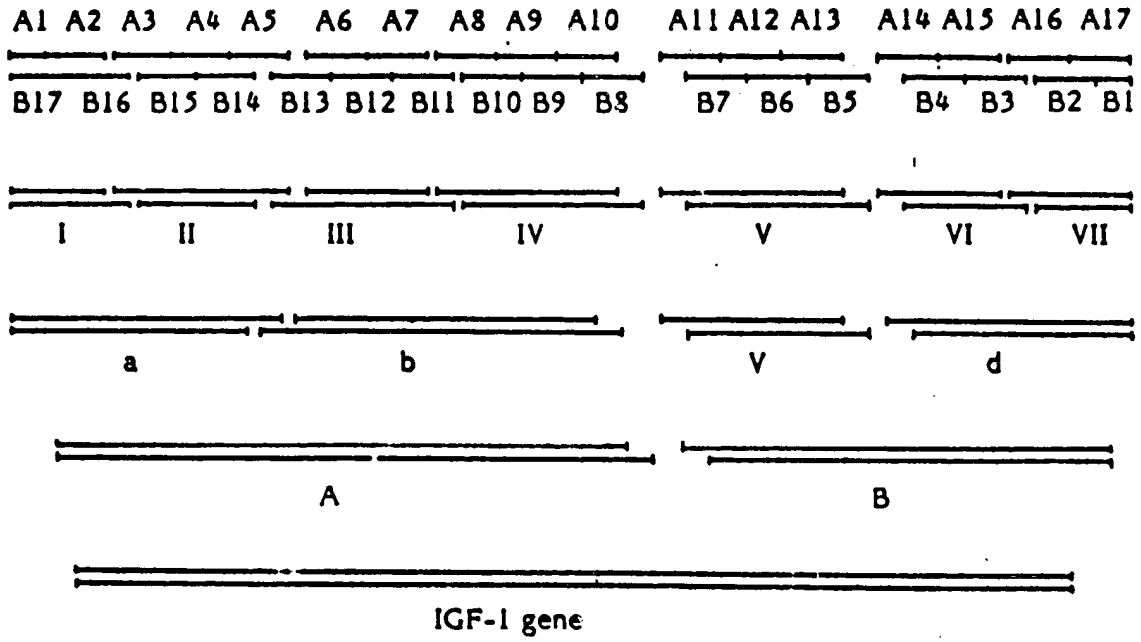


FIG. 13B

INTERNATIONAL SEARCH REPORT

International Application No PCT/SE84/00046

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC 3

C 12 N 15/00

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
IPC 3	C 12 N 15/00
US Cl	435:172, 317

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

SE, NO, DK, FI classes as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category [6]	Citation of Document, [15] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
E	WO, 84/0773 (PHARMACIA AB) 1 March 1984	1
E	WO, 84/0774 (PHARMACIA AB) 1 March 1984	1
A	EP, A3, 1930 (GENENTECH INC) 16 May 1979	1
A	EP, A3, 35384 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) 9 September 1981, see pp 10-12 and 27-29	1-17
P	NY, Acad. Sci Proc. Vol 80 p 697-702 published February 1983 (Löfdahl S.et al) "Gene for staphylococcal protein A"	1-17

[6] Special categories of cited documents: [16]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search [9]	Date of Mailing of this International Search Report [9]
1984-05-09	1984 -05- 16
International Searching Authority [10]	**Signature of Authorized Officer [11]**
Swedish Patent Office	Carl Olof Gustafsson

L.E.